SOME STUDIES ON THE NITROGEN METABOLISM OF SOME ACETOBACTER SPECIES

A THESIS
SUBMITTED TO THE
ALIGARH MUSLIM UNIVERSITY ALIGARH
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN BIOCHEMISTRY
IN THE FACULTY OF SCIENCE

By
WARIS ALI SHAIDA, M. Sc.

1967

Bio-Chemistry Department
MEDICAL COLLEGE
ALIGARH MUSLIM UNIVERSITY
ALIGARH, U.P.

Dated... August 26, 1967.

To whom it may concern.

This is to certify that Mr. Waris Ali Shaida, M.Sc. who had been registered with me for the degree of Ph.D. has completed his work and is submitting his thesis for the award of the degree in Ph.D. in Biochemistry.

The work described in the thesis is original in nature and is being submitted for the first time for the award of the degree and has been independently done by the candidate during the course of his stay at Aligarh.

Intisar Husain

(Intisar Husain)
Professor & Head,
Department of Biochemistry,
Jawaharlal Nehru Medical College,
Aligarh Muslim University,
Aligarh.

T-715

ABSTRACT

Acetobacters or acetic acid bacteria constitute an important group of highly oxidising organisms, oxidising various groups of organic compounds, particularly sugars and sugar alcohols. Catabolism of glucose has been extensively studied but work on the amino acid metabolism and anabolic aspect has received until now only scant attention. The work described in this thesis is related to the Nitrogen Metabolism of some Acetobacter species. Sixteen species of Acetobacter were screened for their ability to deaminate the various amino acids and it was found that:

(1) Out of the sixteen species of Acetobacter tested for their ability to deaminate amino acids, only thirteen produced ammonia from the various amino acids taken as their substrate.

(2) A.aceti gen um (6346) appeared to be particularly promising and was found to deaminate Dl-serine, Dl-norleucine, Dl-leucine, Dl-alanine, Dl-β phenyl-alanine and L-asparagine.

(3) Factors affecting the deamination of amino acids by A.aceti gen um were studied and it was found that:-

(a) The pH optima in case of Dl-alanine and Dl-norleucine were 7 and 7.8 respectively, 7.4 in the case of Dl-phenyl alanine and Dl-leucine and 8 in the case of Dl-serine.

(b) 40°C was found to be the optimum temperature for alanine, phenylalanine, leucine and norleucine deaminases and 45°C for serine deaminase.

(c) With the exception of Dl-serine, where no appreciable difference was observed, in the remaining four amino acids, the amount of ammonia produced as a result of amino acid deamination was much more when the flasks were subjected to shaking than otherwise, when they were kept stationary.

(d) Upto a certain limit, increasing amounts of glucose stimulated to a marked degree the growth of A.aerogenum. The relative level of deaminase activity in each case was found to be related to the amount of glucose present in the medium from which the cells were obtained, and also to the physiological state of the cells of the organism, as expressed by the pH of the culture at the time of the harvesting the cells.

Increasing amounts of glucose at the time of growth, which was also accompanied by a corresponding fall in the final pH of the growth medium, resulted in an inhibition of the deaminase activity to varying degrees in the case of all the five deaminases.

(e) Cells of A.acetigenum, grown for different intervals of time, when tested under the same conditions, were found to vary in their deaminase activity. The best crop of cells of A.acetigenum, possessing maximum activity for the deamination of each of the five amino acids, was obtained when the organism had been grown for 24 hours. The relative deaminase activity of cells obtained after 24 hours growth, and after 48 hours growth, remained almost constant with respect to alanine, phenylalanine and norleucine but showed some variation in the case of leucine and serine. After growth had taken place for 72 and 96 hours, the cells obtained exhibited a considerably reduced deaminase activity for each of the five amino acids tested. The fall in the deaminase activity could not possibly be due to a change in pH since the activity continued to fall, with the age of the culture, even though there was no subsequent appreciable fall in the pH after 24 hours.

(f) Under the conditions of the experiment, it was observed that 3 hours for alanine, 5 hours for leucine, 6 hours for norleucine and 7 hours for serine and phenylalanine represented the time intervals upto which the deaminase activity of the cells of A.anatigenum showed linear proportionality with time. Incubation beyond these time intervals showed a marked decrease in deaminase activity in each case. The percentage deamination, corresponding to these time intervals was found to be 94%, 43%, 30.6%, 54.2% and 40.8% in the case of alanine, phenylalanine, leucine, norleucine and serine respectively.

(g) Cells of A.anatigenum, when stored for various intervals of time, showed a progressive reduction in their deaminase activity. However, in the case of Dl-alanine, the cells retained some deaminase activity even after being kept in the frozen state for a period of three months and the enzyme involved, therefore, appears to be more stable than in the case of the remaining four amino acids.

(4) Both the optical antipodes of alanine, phenylalanine, leucine, norleucine and serine were almost equally deaminated by cell suspensions of A.aceatigenum. The deaminases involved, therefore, appear to be non-stereospecific towards their substrates; or perhaps D-and L forms of the amino acids were being deaminated by different enzymes present in the suspension. However, it is quite likely that the D-form is racemised first to to L-form by amino acid racemase and then gets deaminated. Due to the non-availability of D-amino acids, it was not possible, however, to draw a more definite conclusion from these observations.

(5) The deamination products of the amino acids deaminated by A.aceatigenum were isolated and identified. The keto acids corresponding to leucine, norleucine and phenylalanine were isolated and identified as their 2, 4 dinitro phenyl hydrazones. The keto acids corresponding to alanine and serine, however, could not be isolated.

(6) Indirect evidence was obtained to suggest that pyruvic acid is perhaps produced as a result of deamination of these two amino acids but it gets oxidised near as with the result that it does not get accumulated in the reacting system and could not, therefore, be isolated as its 2, 4 dinitrophenylhydrazone.

the deamination of Dl-alanine by A.aeratigenum was observed.

The results obtained, indicated that inhibition of alanine deaminase occurred to a significant extent when carbonyl group reagents were taken in various concentrations. Of the various carbonyl group reagents tested, sodium bisulphite was able to inactivate completely the enzyme. The other reagents viz. sodium cyanide, hydrazine sulphate and hydroxylamine also exercised a marked effect on deaminase activity except sodium carbazide which could not produce any appreciable inhibition. The results obtained, therefore, provided an indirect evidence of the presence of carbonyl group on the surface of the enzyme which was essential for the activation of the substrate.

The activity of alanine deaminase was also completely lost on the addition of octyl alcohol, caprylic alcohol and toluene.

Of the various metal ions tested, Mg^{++} was without any effect, Zn^{++} inhibited the enzyme to an extent of 38.8%, on the addition of 80 µ moles of $ZnSO_4$ /3 ml. of the reaction mixture, and heavy metals viz. Hg^{++}, Ag^{+},

and Cu^{++} inhibited the deamination of alanine even at lower concentrations. Mn^{++}, however, was able to activate progressively the enzyme at different levels of concentration and activated it to about 45% on the addition of 2 µ moles/ 3 ml. of the reaction mixture.

(8) An attempt was made to investigate the nature of the coenzyme involved in the deamination of Dl-alanine by A.aeatigenum. Only those vitamins and their analogues which were available, were tested. Pyridoxine, pyridoxamine, pyridoxal phosphate and riboflavin phosphate failed to show any noticeable effect on the deamination of Dl-alanine when fresh or two months old cells of the organism, which had lost most of their deaminase activity, were used. Pyridoxamine, pyridoxine and pyridoxal phosphate even showed some inhibitory effect. Riboflavin was not able to exercise a similar effect on the deamination of alanine by A.aeatigenum. Since cell free preparations were not used in these experiments, no satisfactory conclusion with respect to the coenzyme involved could be drawn from these observations.

(9) It was not possible to demonstrate the role of biotin in the deamination of Dl-alanine or Dl-serine.

(10) The metal binding agents also had an adverse effect on the deamination of Dl-alanine and Dl-serine. 8-hydroxy-quinoline and ethylene diamine tetra acetic acid (EDTA)

appeared to be more potent inhibitors than α-α' bipyridyl in the case of both the amino acids. Complete inactivation of alanine and serine deaminases was brought about by 8-hydroxy quinoline and EDTA when they were kept in contact with cell suspensions for 4 hours and 3 hours respectively. However, with α-α' bipyridyl, after 4 hours, this was only about 73% in the case of alanine and 100% in the case of serine. The degree of inhibition in the case of both the amino acids was dependant on the concentration of these inhibitors and also on the time these inhibitors were in contact with the cell suspension. Serine deaminase appeared to be more susceptible to their action than alanine deaminase. These observations suggest that some metals might be involved in the deamination of alanine and serine by A.aceticanum. Inhibition by cyanide also supports this assumption.

(11) The effect of hydroxylamine, sodium bisulphite, semi-carbazide hydrochloride and sodium cyanide on the deamination of Dl-serine by A.aceticanum was also studied. These inhibitors inhibited to some extent the deaminase activity but none of the inhibitors tested could completely inactivate the enzyme even at concentrations as high as 50 μ moles/ 3 ml. of the reaction mixture. Even sodium cyanide, the most potent out of these four, could bring about the inhibition of the deaminase activity to about

66.66% when added in a concentration of 50 µ moles / 3 ml. of the reaction mixture.

(12) Pyridoxine, pyridoxamine, pyridoxal phosphate, cysteine, and anaerobic conditions attained by gassing with nitrogen failed to exercise any appreciable effect on the deamination of both, Dl or L-serine, either with fresh cells, or when two months old cells which had lost most of their deaminase activity were used. Pyridoxal phosphate (PLP), however, was able to activate to a slight extent the deamination of Dl-serine when two months old cells of the organism were used. On the basis of these observations, it can be postulated that in the deamination of Dl-serine perhaps two enzymes, D serine deaminase and L-serine deaminase are involved. The former seems to be PLP dependant, and is inactivated in the presence of carbonyl group reagents, while the latter is not. This would also explain about 50% inhibition of serine deaminase activity in presence of carbonyl group reagents. Further, pyridoxine or pyridoxamine were not found to be able to replace pyridoxal phosphate.

(13) Evidence has been presented to suggest that the deamination of Dl-alanine by A.acetigenum might be catalyzed by an amino acid oxidase system.

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CONTENTS

INTRODUCTION

CHAPTER I

THE ACETIC ACID BACTERIA

The microbial acetification of alcoholic liquids has been known due to its duel behaviour. On one hand it can cause the spoilage of wines, beers and other alcoholic beverages which can be regarded as its evil aspect, and on the other hand it has been of great value to mankind since by experience it has been shown that one of the products of acetification of wine was vinegar which has been known to civilization since the dawn of history for its medicinal, preservative and condiment value. The primitive art of vinegar production has been known for more than hundred years and Boerhaave 1) in 1737 designed the first vinegar generator but its microbial nature could be known to the world only about 100 years back after the great controversies between Liebig, Kutzing and Pasteur. Berzelius in 1820 proposed that the cause of acetification of alcoholic beverages was the acetic acid itself which was enclosed in small pores of membranous skin present in solutions undergoing acetification. Persoon 2) named the skin as "Mycoderma" but did not assign any role to it in the acetification process and the mechanism of acetification remained un-understood till Kutzing 3) proposed that mycoderma or "mother of

vinegar" was composed of minute living organisms and the acetification could be brought about only by living mycoderma which he named as "Uvula aceti". His idea did not receive any support in the beginning from any of his contemporaries. Liebig (4), a leading chemist of that time, strongly contradicted Kutzing's finding and put forward his iews that acetification of wine was not a physiological process but that it was identical to the oxidation of alcohol to acetic acid by platinum black and the function of mother of vinegar was merely to absorb oxygen for such an oxidation. Later, Pasteur's great researches in 1860's on wine and vinegar finally supported Kutzing's ideas based on physiological basis. Pasteur called mother of vinegar as "Mycoderma aceti" which was considered as the primary cause of most fermentations. He showed that this organism could be grown upon neutral medium supplemented with alcohol, and absorbed oxygen, producing acid during growth. Later, Pasteur's theory of acetification of wine was confirmed by L.Wurm (5) who also supported the idea that formation of vinegar from alcoholic liquids was due to an organized ferment. Hansen (1887-1893) and Brown (1888) recognized that most acetifying organisms were bacteria and both the workers described additional species of vinegar producing bacteria(6,7,8). A definitive study of the group was made by Beijerinck in 1899 who dropped the term mycoderma and coined the name "Acetobacter" for them in 1901. He recognized them as a group with many morphological

and physiological similarities. Many additional species viz. Bacterium acetosum, Bact.acetigenum, Bact.ascendens, Bact. oxydans, Bact.orleanse and Bact.xylinum etc. were later isolated from malt, beer, wine, and vinegar by Henneberg (9). Baker, Day and Hulton (10) in 1912 isolated 150 microorganisms from ropy materials and showed that eleven of them produced ropiness in beer. Due to their close resemblance they were given the collective name of Bacterium aceti viscosum; Bact. rancens and Bact.melanogenum were isolated by Beijerinck (11). Kluyver and Leeuw (12) discovered Acetobacter suboxydans (12) which brings about an incomplete oxidation of a number of organic compounds. A.capsulatum, another rope forming organism, was reported by Shimwell (13) in 1936. Cosib, Tosic and Walker (14), in 1941, isolated a new species which caused acid production and turbidity in beer and named it as Acetobacter turbidans, which could cause spoilage of beer (15). Later, Tosic and Walker isolated other species named Acetobacter mobile (16) and Acetobacter acidum-mucosum and described their biochemical properties and other important characteristics (17).

General Characteristics of Acetobacter

Acetobacter belongs to the family Pseudomonadaceae. All members of this group of bacteria are bacillus in nature and therefore, the cells are rod shaped to ellipsoidal, occurring singly, in pairs, or in short or long chains.

Involution forms are curved, elongated, club shaped or some time branched. The cells occur singly, in pairs or in chains. Non-motile or motile with polar or peritrichous flagella. Young cells, gram-negative, old cells gram-variable, generally catalase positive with the exception of A.paradoxum and Visser Hooft's A.peroxydans. They are strictly aerobic in nature and possess the characteristic property of oxidising alcohol to acetic acid. This property is also possessed by some of the fluorescent Pseudomonas but they are capable of bringing about oxidation only in buffered media, while acetobacters can bring about rapid and complete oxidation of alcohol to acetic acid under extremely acidic conditions. The important differences between them can be summarized as follows:

1. Pseudomonas can not grow in a medium more acidic than pH 5 while Acetobacters can survive and grow even at a lower pH.
2. Pseudomonas do not form surface films like Acetobacters.
3. Most of the species belonging to the Pseudomonas group produce characteristic green, blue, or fluorescent pigments which are not found in Acetobacters except in case of A.melanogenum and A.roseum where brown and pink pigments are produced respectively.
4. Pseudomonas attack peptones and proteins vigorously while Acetobacters are not able to do so.

Acetic acid produced from alcohol or glucose may get accumulated due to an incomplete oxidizer like A.suboxydans A.xylinum, A.melanogenum or get over oxidised to CO_2 and water by complete oxidisers like members of the peroxydans and oxydans groups in Frateur's classification of Acetobacter. Acetobacter can oxidise a number of organic compounds and the common oxidation products are vinegar or acetic acid from ethyl alcohol; gluconic acid, ketogluconic acid from glucose; dihydroxyacetone from glycerol and sorbose from sorbitol. Some can also produce acid from dextrose, but none can attack starch, glycogen or inulin. Nutritional requirements vary from simple to complex. The best medium recommended upto now is yeast extract and glucose. One of the striking properties of some Acetobacter species is their ability to synthesize cellulose. Some other species like A.turbidans, A.viscosum and A.capsulatum synthesize polysaccharidic (non-cellulosic) capsules (18,19). Kulka and Walker (20) regard the ability to form capsules as one of the important properties of this genus.

Classification

Attempts have been made from time to time for the species classification of this genus on the basis of morphological, nutritional and biochemical properties but none of the methods proposed so far has proved satisfactory. K.Miyaji (21), a Japanese worker, in 1924 isolated some thirty varieties of acetic acid bacteria and classified them into three groups on morphological and physiological grounds. Several other workers have isolated and named various types of Acetobacter but it has been seen that the same Acetobacter has often been given different names and different cultures have some times been given the same name. For example, A. aceti Hansen is not the same as A.aceti Beijerinck and the two organisms named A.ascendens have quite different properties. A.pasteurianum on continued culturing lost some of its properties and the resulting strain behaved like A.rancens. Henneberg's classification of these bacteria into four groups based on their habitat is also not tenable since the same species occur in very different habitats; he also created a number of new species. Similarly Walker et al have described a few new species. Visser't Hooft's physiological types were developed into a system by Kauchn (22). This scheme reduced the number of species considerably, as was necessary, and provides a useful working key to the more common species.

In this scheme, as is apparent from table No.1, Acetobacters are divided on the basis of oxidation of acetate to carbon dioxide and water. Further division into groups was done on nutritional and morphological basis. Vaughn's dichotomous division was further supported by the observations of other workers. On the basis of flagellation and acetate oxidation, Leifson (23) has suggested that the genus be split into two: Acetobacter, comprising the species that oxidise acetate and have peritrichous flagella, if any; and acetomonas, consisting of species which cannot oxidize acetate and are polarly flagellated. Similar grouping is also suggested by the nutritional experiments of Rainbow, et al.(24,25) whose lactaphilic and glycophilic groups correspond to the Acetobacter and the Acetomonas respectively, these in turn correspond to the two physiological types of Vissert Hooft. Likewise the work of Asai and Shoda (26) and Shimwell's work (27) on flagellation all support Leifson's conclusion and favour the retention of the dichotomous division of Vaughn. However, the Japanese workers prefer the name Gluconobacter to Acetomonas because of the marked capacity of organisms of the new genus to accumulate gluconic acid in glucose media. In his detailed system of species identification, Frateur (109), having recognised the dichotomy of types of acetic acid bacteria, proceeds to divide those which over oxidize acetate

into peroxydans, mesoxydans, and oxydans groups, while those which do not do so form his suboxydans group. Catalase character, the ketogenic capacity, and the ability to oxidize acetate to CO_2 and water have been used to assemble these organisms into four groups which are then sub-divided into various species according to their ability to oxidize glucose, to utilise NH_4-N as the sole source of nitrogen in Hoyer's medium and to form pigments and cellulose. Recently, Rainbow (28) has summarized all these systems of classification in one chart given in table No.II. According to Shimwell, the classification of strains of the genus Acetobacter proper as distinct from Acetomonas(Leifson)remains not only unsatisfactory but is impossible on any criteria because of the facility with which strains of Acetobacter proper undergo mutation. Shimwell's paper (27a) adduces striking evidence of this mutability which has also been attested, particularly with respect to the power to secrete starch or cellulose, by other workers (89,89a,) and by Shimwell himself in an earlier paper (29) in which he suggests that there may be only one or perhaps two species of Acetobacter proper and that members of the suboxydans group are of different phylogenetic origin. The remarks of van Niel and Stanier as quoted by Rao (52) are very approriate here:

"In an attempt to subdivide the organisms belonging to one natural group of bacteria into species, one would have to create as many species as there are organisms which differ in sufficiently fundamental characteristics, regardless of the existence of intermediate types. It depends entirely on the 'scientific tact' of the investigators to decide which characteristic shall be deemed worthy of the designation "sufficiently fundamental".

It, however, seems that the system of Frateur is the most comprehensive and the best available now but there are a number of anomalies in the results and incompatibilities in the grouping of the species as discussed above and it is only for the taxonomic experts to evaluate the utility of the various systems proposed.

TABLE I

Key to the species of Acetobacter (after Vaughn)

Group I OXIDISE ACETIC ACID TO CARBON DIOXIDE AND WATER			Group II DO NOT OXIDISE ACETIC ACID			
(A) Utilize Ammonium salts as a sole source of nitrogen	(B) Do not utilize ammonium salts as a sole source of nitrogen		(A) Form pigments in glucose media.		Do not form pigments.	
	1. Forms a thick zoogloeal cellulose membrane on the surface of liquid media	2. Do not form a thick membrane on liquid media	1. Dark brown to blackish pigment	2. Pink to rose pigment	1. Optimum temp. between 30° and 35°	2. optimum temp. between 18°-21°
(1) A. aceti	2. Acetobacter xylinum	3. A. ceto bacter rancens.	4. Acetobacter melanogenum	5. Acetobacter roseum	6. Acetobacter suboxydans	7. Acetobacter oxydans
		3a. A. pasteurianum				
		3b. A. kuetzingianum				

T A B L E - II

Summary of evidence that Acetic acid bacteria comprise two taxonomically distinct groups.

(After Rainbow, C: Progress in Indust.Microb.Vol.3,48 (1961)

References	Groups recognized	
Vaughn,R.H. (1942) Wallestein Lab.Comm. 5, 5.	Group I oxidize acetate	Group II Do not oxidize acetate
Frateur,J.(1950) La Cellule,53,287.	Peroxydans,mesoxydans, and oxydans groups.	Suboxydans group.
Leifson E.(1954) Leeuwenhoek ned Tijdschr,20,102.	Acetobacter:non motile or peritrichous flagella; oxidize ethanol, through acetate,to CO_2, and water;oxidize lactate.	Acetomonas:Non motile or polar multitrichous flagella, do not oxidize acetate and lactate.
Shimwell, J.L. (1958) Leeuwenhockned Tijdschr 24, 187.	Acetobacter: as Leifson (1954).	Acetomonas (Leifson) n motile or possessing one or more polar flagella;oxidize ethanol only as far as acetate;do not oxidize lactate.
Asai T., and Shoda, K (1958) J.Gen.Applied Microbiol,Japan, 4, 289.	Acetobacter as Leifson (1954) relatively powerful oxidizer of ethanol to acetate.	Gluconobacter:A, Acetomonas Leifson, relatively powerful oxidizer of glucose to gluconate.
Rainbow, et al (1953),J.Gen. Microbiol,9,371 J.Gen.Microbiol 15, 61 (1956).	Lactaphiles:grow well on lactate media; simple growth requirements;resting cells transform amino acids related to glutamate to other ninhydrin reacting substances.	Glycophiles grow well on glucose but not on lactate;relatively complex growth requirements; resting cells transform amino acids related to glutamate feebly if at all.

TABLE - III

Properties of Lactophilic and Glycophilic Acetobacters

Lactaphiles	Glycophiles
1. Grow well on lactate, but relatively poorly on glucose.	1. Grow well on glucose and certain sugar alcohols, but not on lactate.
2. Frequently capable of utilizing NH_3 as sole source of nitrogen on lactate media.	2. On otherwise suitable media, utilize NH_3 as sole source of nitrogen only slowly and feebly.
3. Transform glutamate, aspartate and proline to other ninhydrin reacting substances.	3. Possess little or no power to transform glutamate, aspartate, and proline.
4. Usually do not require exogenous supplies of growth factors.	4. Require exogenous supplies of certain growth factors.

Nutrition and Metabolism

Very little work has so far been done on this aspect of Acetobacter. In early studies of acetic acid bacteria, the nutritional requirements varied from as simple a medium as ammonium salts (Hoyer's medium) to more complex medium containing yeast extract, malt extract, beer and the like. It was by the use of defined and semi-defined media that led the workers later to know the detailed nutritional requirement of Acetobacter species. It has now been shown that Acetobacters require certain vitamins of B group as growth factor in addition to carbon and nitrogen sources. These growth factors are paraaminobenzonic acid, nicotinic acid, pantothenic acid for A.suboxydans (30A) and A.rancens (31). A melanogenum and A.oxydans require thiamine in addition to the three mentioned vitamins. Biotin is also reported to be required for growth of A.ascendens, A.pasteurianum and A.acidum-mucosum (32). Pantothenic acid requirement of Acetobacters attracted much interest of the workers mainly for determining the structure of CoA and the reaction sequence of its synthesis. Pantothenate is also required by A.ascendens, A.capsulatum, A.pasteurianum, A.turbidans (32) and by A.gluconicum (24). The requirements of these growth factors are fulfilled by the use of complex media containing yeast extract. Much attention has not so far been laid on the amino acid requirements for this group. Many Acetobacters require preformed amino acids and vitamins in the growth medium while

some, like A.melanogenum, can synthesize all the amino acids needed for growth from ammonium salts but require several vitamins (31). Carbon source plays a very important part in the metabolism of acetic acid bacteria. Sugars or related substances are shown to be required to start growth after which ethanol may be used as an additional source of carbon and energy. The studies of carbon compounds led Rainbow, Niston and Brown (24,25) to recognize acetic acid bacteria into lactaphilic and glycophilic types. C. Rainbow (28) has recently given the criteria of distribution of these two types as is given in Table No. III. They concluded that lactaphiles have very simple nutritional requirements and are, therefore, well equipped enzymically and can synthesize their requirement materials from simple substance whereas Glycophiles are not well equipped with enzymes and can not synthesize the essential requirements from simple substances. However this classification is not rigid as there are certain strains that can not be fitted exactly in one or the other group.

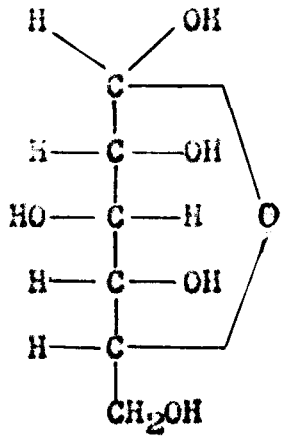
The existing literature that has so far been cited indicates that Acetobacters are highly oxidising organisms, oxidising various groups of organic compounds, particularly sugars and sugar alcohols. Catabolism of glucose has been extensively studied but work on the amino acid metabolism and anabolic aspect has received until now only scant attention.

Many species of Acetobacters can form a variety of compounds from the oxidation of glucose depending upon the conditions of the experiment and the species used. Thus glucose (33,34) can be converted to gluconic acid, ketogluconic acid, glucuronic acid, succinic acid, fumaric acid, lactic acid, oxalic acid and acetic acid.

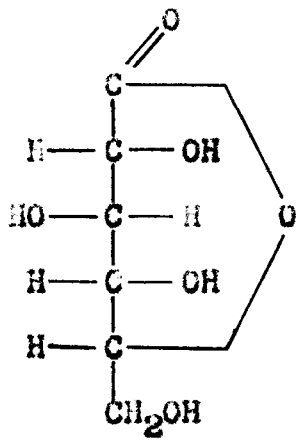
Butlin (35) has reported that A.suboxydans resulted in the conversion of glucose to CO_2 only in buffered media, carbon dioxide production varied with the age of the culture and the presence of acid in the growing medium. Old cells due to production of excessive amount of acid could not produce CO_2 from glucose and by addition of acid the effect becomes even more pronounced (35,36). It was, therefore, concluded on the basis of these observations that A.suboxydans possesses two enzyme systems with respect to glucose oxidation; one responsible for co version of glucose to CO_2 is acid sensitive and cytochrome linked, the other acid stable component is related to the formation of gluconic acid and ketogluconic acid (35).

It was also shown by Fewster (37) that washed cells of A.suboxydans produced CO_2 and D-arabonate from 2 ketogluconic acid. 5-Ketogluconic acid did not produce CO_2 unless some oxidisable substrate was present. The absence of chalk in the growth medium showed lesser oxidative ability but still showed

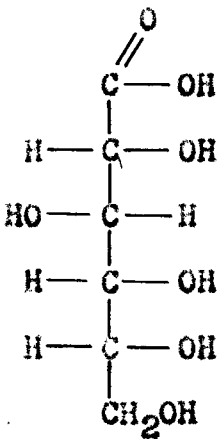
the power of oxidising glucose to gluconate. Thus an acid sensitive enzyme for CO_2 production from glucose was established. Like glucose other aldose sugars like galactose and arabinose are oxidised to galactonic acid and arabonic acid respectively (66). The production of gluconic acid from glucose takes place through the formation of glucono-δ-lactone as follows. Later gluconic acid gives 5-ketogluconic acid or 2-ketogluconic acid.



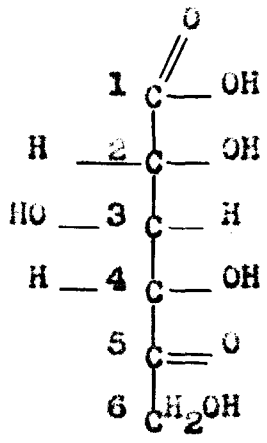
Glucose



Glucono lactone

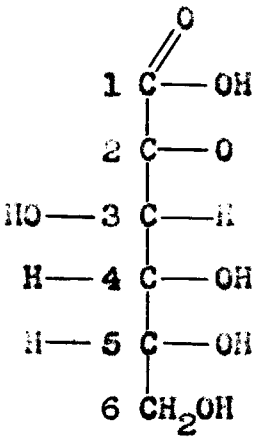


Gluconic acid



5-Keto-gluconic acid

Or



2-Keto-gluconic acid

A.acetigenum, A.gluconicum, A.orleanse, A.turbidans, A.viscosum, A.xylinum and A.kuetzingianum(38) produce both 2-and 5-keto gluconic acid while others like A.ascendens, and A.xylinoides etc. form only 2-ketogluconic acid (39). Accumulation of these ketonic acids depends on the presence of $CaCo_3$ in the growth medium. A.melanogenum produced even a third reducing (ketonic) compound, 2,5-diketo gluconic acid along with the usual 2-, and 5-keto gluconic acid (40) and this diketo acid was found to be the precusor of the characteristic brown pigment (41) produced by this organism.

Primary steps in the oxidation of glucose upto the formation of 2,5-diketo gluconic acid have been worked out by Katznelson et al. (41). They found that 2-keto gluconic acid, gluconic acid and glucose require 1,2, and 3 moles of oxygen respectively for their conversion to 2,5-diketo gluconic acid. The reaction sequence presumably is as follows.

Glucose ⟶ Gluconate ⟶ 2-Ketogluconate ⟶ 2,5-diketo-gluconate

The enzyme systems controlling these reactions have not yet been worked out. 5-Ketogluconate in A.melanogenum was not further metabolized but in other species it was metabolized by an unknown pathway to D-tartaric acid and oxalic acid (42). In recent studies on the aerobic metabolism of 5-keto gluconic

acid by intact cells of A.suboxydans (43) evidence was obtained that small amount of pyruvic acid was also formed in the medium besides other products. Conversion of glucose to tartronic acid was found to be either due to slow spontaneous reaction or due to enzyme action of A.acetigenum as reported by Walker et al (44,45).

Horecker's Pentose cycle has been reported to be operative in A.suboxydans by Hauge et al.(46,47) and this finding was confirmed by Kitos et al (48) who reported that glucose and gluconate oxidation proceeded substantially via the pentose pathway. However TCA cycle enzymes were not found to be active in A.suboxydans. It possessed only fumarase and aconitase of the TCA cycle enzymes. On the other hand, A.pasteurianum (49) A.turbidans (50) and A.peroxydans (51) have been reported to oxidise acetate, pyruvate, succinate, malate, and fumarate. As the resting cells of A.pasteurianum (52) and cell free extract of A.aceti oxidized all the TCA cycle intermediates, this led King et al (49) to believe that this cycle is operative in these organisms. And recently, C.Rainbow (28) has pointed out that on the basis of carbohydrate metabolism, Acetobacters can be classified into two main groups: (1) those in which both the pentose and TCA cycles are in operation. Members of this group belong to Frateurs Mesoxydans and oxydans class. (2) in this group carbohydrates are degraded by Pentose cycle; and TCA cycle is not involved at all; the members of this group correspond to Frateurs Suboxydans group but this is to be seen whether

these characteristic features of these groups remain to be as such in future too.

Some Acetobacters have also been reported to possess many of the enzymes active in glucose fermentation. Thus, A. suboxydans and A.rancens var pasteurianum could convert hexose diphosphate to triose, acetaldehyde, and acetate (53).

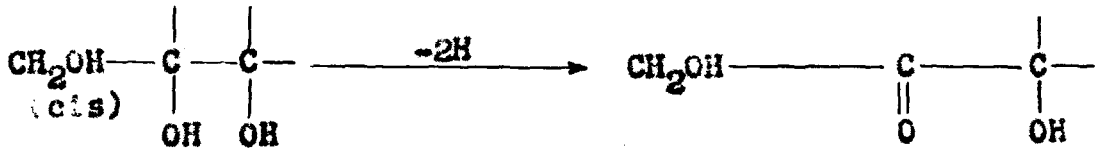
Glyceraldehyde-3-phosphate dehydrogenase was not present in A.suboxydans. This together with the fact that acetate is not formed from glycerol indicates that E.M.P. fermentative path way is not active in A.suboxydans (54) though Kitos (55) has given an indirect evidence for the possibility of E.M.P. route along with pentose cycle in A.suboxydans but any strong evidence for its operation in Acetic acid bacteria is not yet available.

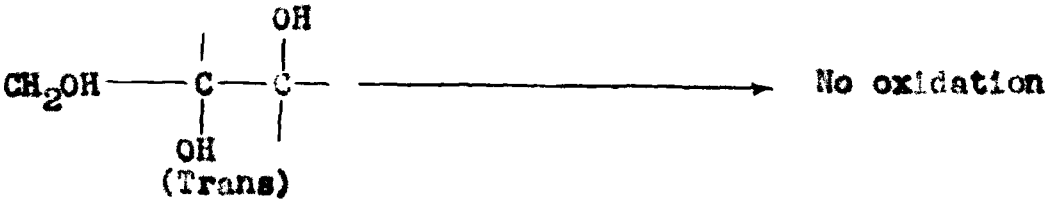
Metabolism of carbohydrates by other than the above mentioned routes also takes place and the unpublished work of Katznelson and Wood as quoted by Kovachevich and Wood (56) indicates that 2-keto-3-deoxy-6-phosphogluconate aldolase and 6-phosphogluconate dehydrase enzyme of Entner-Doudoroff pathway is present in A.melanogenum. An acceptable support for the operation of dicarboxylic acid cycle is also given by Tanenbaum (57) in case of A.peroxydans which oxidized acetate, succinate, fumarate, malate, oxaloacetate but not citrate, iso-citrate and $\mathcal{L}$ -ketoglutarate (57).

General Catabolism of carbon compounds.

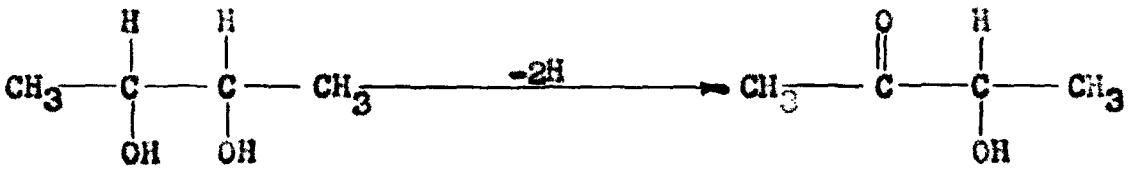
Acetic acid bacteria oxidise a number of open and closed polyols and other organic compounds and the oxidation of ethanol to acetic acid is one of the most classical examples of oxidation by this genus. The complete oxidisers of the substrate to carbon dioxide and water are not industrially important but those which result in partial oxidation are of great practical value due to production of valuable substances like gluconate, 2-ketogluconate, 5-ketogluconate, 2,5-diketo-gluconate, sorbose etc. Similarly, production of rare sugars and specific oxidation of steroids is also brought about by incomplete oxidizers (58).

Bertrand first reported the production of a ketogenic substance sorbose by the oxidation of sorbitol by A.xylinum. The other important oxidizers of sorbitol to sorbose are A.suboxydans, A.xylinoides (59). Later, Bertrand after studying the oxidation products of a number of polyhydric alcohols (polyols), founded a generalization called 'Bertrand's rule for polyols oxidation. This rule states that secondary alcoholic group of polyol is oxidized to a ketonic group if it is situated between a primary and another secondary alcoholic group cis to the susceptible group.

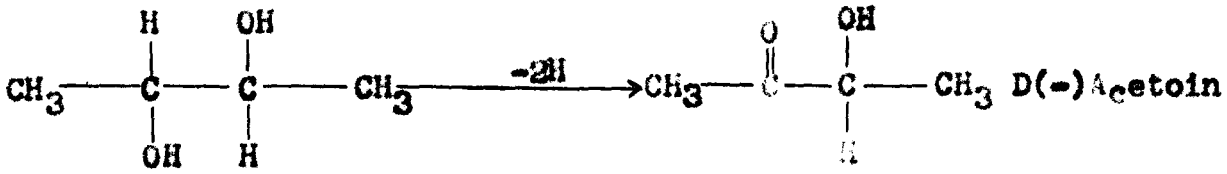
Thus 

but 

Thus, in accordance with this rule, A.suboxydans is reported to oxidize mannitol to D-fructose, D-perseitol to D-perseulose, D-arabitol to D-xylose, erythritol to erythrulose and D-gluconate to 5-ketogluconate. Cases where this rule does not hold good and the secondary alcoholic groups are dehydrogenated are glycerol which gives dihydroxy acetone, 2,3-butanediol which gives acetoin and meso-hexanediol which gives rise to.D-Hexane-3-one-4-ol. It was later reported by Risseghem (60) and underkofler (61), working respectively on meso-hexane diol and erythritol, that D-carbon atom was oxidized when both D and L carbon were present in the same molecule. The same is true for isomers of 2,3-butane-diol as follows:

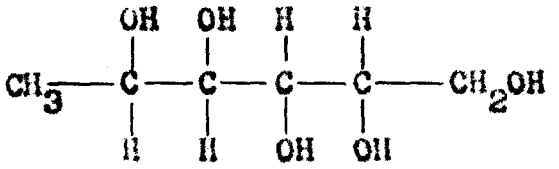


Meso-butanediol L (+) Acetoin



D(-)butanediol

L(+)butanediol

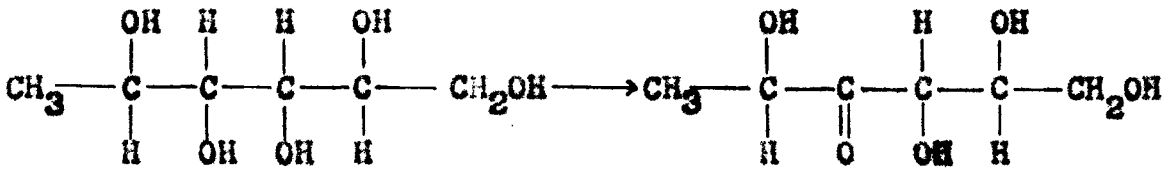
similarly L-rhamnitol  was not

oxidized though it fulfills all the requirements of Bertrands rule. Only D form is oxidized and hence Bertrands rule was

D-rhamnitol Rhamnose

modified by the fact that the pair of cis secondary alcoholic groups must have a D-configuration as reported by Hann, Tilden and Hudson (62). However, this has got also certain anomalies as is seen in case of the oxidation of L-fucitol and other 6-de oxysugar alcohols by A.suboxydans (63,64). These substances were oxidized even in the absence of "Bertrand — Hudson rules".

requirement. The oxidation occurs as follows.



L-fucitol L-fuco-4-ketose

In order to make"Bertrand — Hudson Rule" applicable in these cases too, Richtmeyer et al.(63) suggested that the rule can still hold good in case of sugars having the following configuration when carbon 3rd from left will be oxidized. In other words if

CH_3-CHOH group is regarded as an elongated form of CH_2OH where one of the hydrogen of primary alcoholic group is replaced by a CH_3 group. In this way the stereo-chemical requirement of "Bertrand-Hudson Rule" is again fulfilled for oxidation of polyols.

The generalization for the oxidation of substrates is undoubtedly applicable in most of the cases but is complicated by species differences as is reported by Han et al.(62).

And this can well be seen in case of A.suboxydans and A.xylinum, where the former oxidises polyols having cis OH groups and D configuration and later needs only cis-hydroxyls.

Oxidation of cyclitols

Closed chain polyols are also oxidised by acetic acid bacteria. This led Posternak to resolve the problem of the structure of inositol and related cyclitols. Posternak's work supported the configuration of meso-inositol (66) and it was reported that A.suboxydans oxidises L-inositol to give a keto group at C_3 and on further oxidation w th $KMnO_4$ and Na_2CO_3 dl-saccharic acid is obtained establishing the structure of the ketone. Posternak with his coworkers (67) working with cyclohexane-1,2,3-triol formulated a rule that cis-vicinal OH group are necessary for oxidation.

Nagasanik et al (68,69) after studying the oxidation of cyclohexane hexol and Pentol concluded that there must be two minimum requirements for a cyclitol to be oxidised; (a) only polar hydroxyls, those which project above or below the puckered plane of the ring; and (b) the carbon atom in the meta position to the one with polar hydroxyl (counter clockwise if north polar and clockwise if south polar) must have an equatorial hydroxyl.

But according to Posternak (70) this generalization does not hold good in most of the cases as epi-inositol, dl-epi-meso inosose, tetrols, triols and diols were not oxidised according to Magasanik-Chargaff rule.

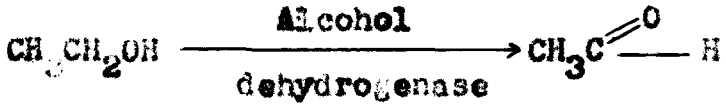
Rare inositol, quercitol, inositol methyl ethers and 2-cyclohexenetetrols have also been reported to be oxidised by A.suboxydans ATCC 621. Oxidation of Neo-quercitol and methyl ether inosose are described for the first time. Recently Posternak reported (72) the role of oxidation of cyclitol by A.suboxydans. He showed that in contrast to the rules of Magasanik et al. only the alloinositol, mucoinositol and cis-quercitol were oxidised. The products were corresponding ketones. Still more work is required on this aspect till any final generalization can be drawn for cyclitol oxidation by Acetobacter species.

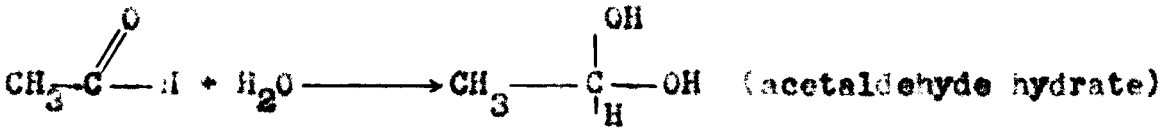
Ethanol Oxidation mechanism.

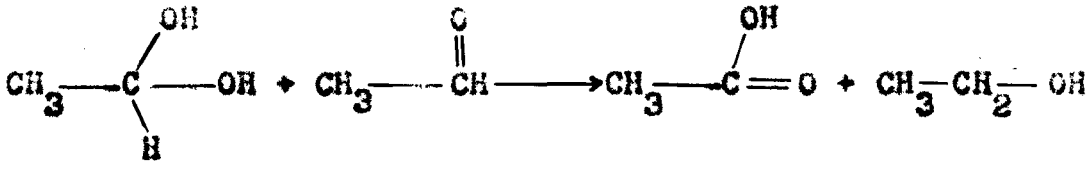
Acetaldehyde has been found to be the intermediate product during the course of oxidation of alcohol to acetic acid. Indeed, industrial experience has taught that acetaldehyde will accumulate during vinegar production when aeration is inadequate.

The production of acetic acid from acetaldehyde is brought about by two mechanisms. The first mechanism was proposed by Neuberg and Windisch (73) and will operate when

the condition is anaerobic and the medium is not acidic. The process starts by the formation of acetaldehyde hydrate which donates its hydrogen to other acetaldehyde molecule by NAD which links together the two enzymes, alcohol dehydrogenase and aldehyde dehydrogenase (74). This results in a Cannizaro reaction producing one mole of each acetic acid and ethanol. The latter is retransformed to acetaldehyde which in turn will dismutate in the manner illustrated above and the cycle of alternate oxidation and dismutation is repeated until the ethanol is completely depleted. This mechanism has been reported to operate in A. nascendens, A. pasteurianum and A. xylinium (75) as follows.







The other mechanism as proposed by Butlin (76) and based on strictly aerobic conditions is most widely accepted. According to this mechanism, alcohol is dehydrogenated by NAD dependent alcohol (79,80,81) dehydrogenase to give acetaldehyde which after being activated by acetaldehyde dehydrogenase

(80,81,82) transfers its hydrogen to NAD or NADP. And finally hydrogen is presumably transferred to molecular oxygen through the mediation of cytochrome system, the presence of cytochrome has actually been reported in Acetobacters by various workers (77,78). Chin (78) has reported that cytochrome a1, a2, and a4 are present in A.peroxydans. Dehydrogenation of acetaldehyde occurs only in its hydrated form. NAD and NADP which are co factors for alcohol and aldehyde dehydrogenase (79-83) respectively, have recently been reported by Atkinson and William F.Sevate (84) to be actually reduced at the expense of oxidation of alcohol and acetaldehyde. After removal of particulate oxidase system, EtOH was specific for NAD and superficial fraction contained the NADP dehydrogenase. The scheme given by them is as follows:

$$(1)\quad CH_3CH_2OH + NAD \underset{\text{dehydrogenase}}{\overset{\text{Alcohol}}{\rightleftharpoons}} CH_3CHO + H^+ + NADH$$

$$(2)\quad NADH + O + H^+ \xrightarrow{\text{cytochrome system}} NAD + H_2O$$

$$(3)\quad CH_3CHO + NADP + H_2O \underset{\text{genase}}{\overset{\text{Aldehyde dehydro}}{\rightleftharpoons}} CH_3COOH + NADPH + H^+$$

$$(4)\quad NADPH + O + H^+ \xrightarrow{\text{cytochrome system}} NADP + H_2O$$

Some Japanese workers (85) in 1960 extracted alcohol dehydrogenase and acetaldehyde dehydrogenase from Acetobacter species and they found that cytochrome was bound to particulate fraction. Aldehyde dehydrogenase was purified

and inactivated by dialysis, activated by NADP, Co^{++}, Mg^{++} or Mn^{++}, slightly inactivated by ethylene diamine tetra acetic acid (EDTA) and pyrophosphate, strongly by cyanide, $NH_2.NHCO.NH_2$, $NaHSo_3$, As^{5+}, CH_2I COOH, p-chloromercuribenzoate and NH_2OH. A new coenzyme dependent aldehyde dehydrogenase was isolated and purified by Takeyoshi Nakayama (86) and was called acetaldehyde ferricyanide reducing enzyme. This catalyses electron transport from acetaldehyde to $Fe(CN)_3^{3-}$, methylene blue or 2,6-dichloro indophenol but not to any known coenzyme.

Polysaccharide synthesis

Many of the species of Acetobacter synthesize cellulosic and non-cellulosic polysaccharides. Cellulose synthesis is well demonstrated in A.xylinum. The cellulose is produced in the form of thick, tough, leathery pellicle on the surface of growing culture. The presence of tannic acid in growth medium produces a heavy insoluble mass; in world war I it was even proposed to make "bacterial leather" in this way. A. acetigenum and A.xylinoides which are regarded as the varieties of A.xylinum are also found to produce cellulose. The former also gives rise to celluloseless mutant (87). Celluloseless mutant of two strains of A.acetigenum and A.xylinum var africanum have also been reported by Steel and Walker (88) Later, the same workers undertook a comparative study of cellulose producing and non-cellulose producing mutants obtained by various methods including natural and induced mutation (89).

A.kuetzingianum and A.pasteurianum have also been reported to synthesize cellulose; but this lacks confirmation (90,91). However, recently Dudman (92) has shown that upto 25% added sugar in medium could be converted to cellulose by A.kuetzingianum and A.pasteurianum.

The nature of pellicle was not known in the beginning but later studies confirmed that it is cellulosic in nature as is confirmed by chemical tests (91,93,94) infra red (94,95) and X-ray studies (91,93,94,96,97). Similarly, studies with electron micrograph (94,98), degradation of pellicle to glucose (91,93,94) and cultivation of cellulose degrading bacteria on pellicle (99) clearly indicated the cellulosic nature of the pellicle. The nature of the carbon source in the growth medium affects cellulose synthesis. Disaccharides and pentoses have been found to be poor precursors, whereas glucose, fructose, arabitol, glycerol and mannitol are the most suitable one. Nitrogen source also affects cellulose synthesis (100). Paraaminobenzoic acid was necessary for growth as well as for cellulosic synthesis in A.xylinum (100) Ammonium sulphate, asparagine plus glutamic acid were equivalent nitrogen sources in the production of cellulose by A. acetigenum (92).

The mechanism of cellulose biosynthesis by Acetobacters has been worked out by numerous workers but is still unsolved.

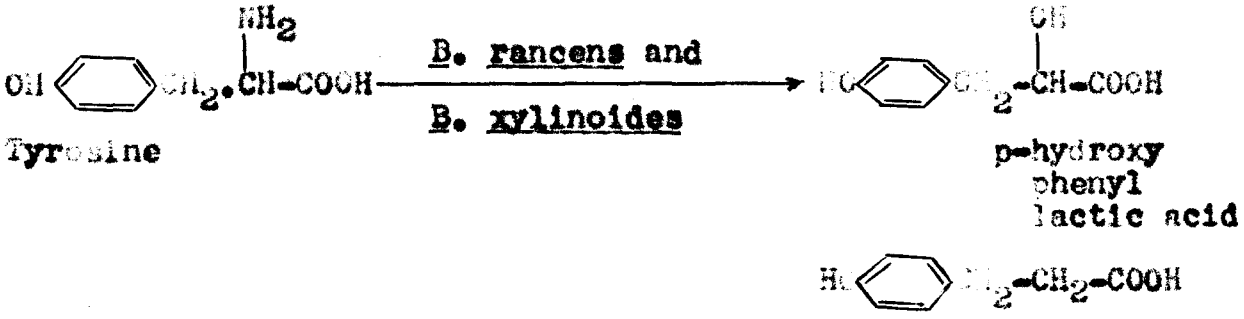
As the cellulose fibrils are excreted out side the bacterial cells, the soluble precursor that polymerizes to form cellulose has to be excreted. Schramm et al (101) in 1957 showed that glucose units polymerize to synthesize cellulose and this was shown by incorporating C^{14} glucose in growth medium and detecting the isotope in position 6 of the constituent glucose units of the synthesized cellulose. When the cells take up C^{14}-glucose, the isotope is transferred to cellulose - the C_6 position almost 100 percent, the C_1 and C_2 less completely, due to being partly oxidized away. This shows that polymerization of glucose units is the source of cellulose but the precursor is not an oligosaccharide nor a sugar phosphate, although Walker et al. (102) and Ziegler and Weigl (103) have shown that these are excreted to some extent during growth, and are involved in cellulose synthesis. Colvin (104) and Khan (105) have reported the formation of micro-fibrils insoluble in alkali and apparently true cellulose by mixing the alcoholic extract of A.xylinum with the ultra filtrate of the cells. The extract contained a lipid-bound glucose complex. The alcohol of the lipid was aliphatic in nature of approximately $C_{28}H_{58}O_3$ molecular entities. The ultra filtrate contained an enzyme which could bring about the synthesis of cellulose microfibrils from the precursor. Their conclusion has been substantiated recently for A.acetigenum (106). Thus the cells after taking glucose of the medium during incubation combine it with lipid to form

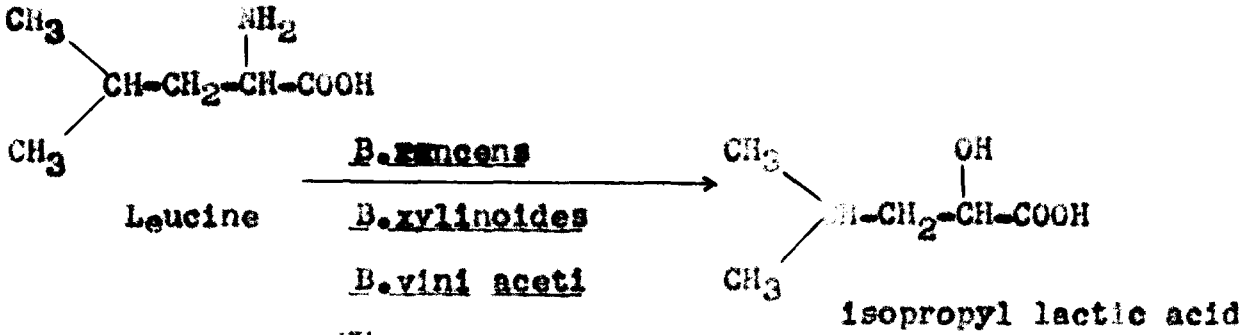
the precursor at the cell membrane, and excrete it together with the enzyme that after removing hexose unit orient them into fibrils. This excretion may be connected with small modified areas on the cell surface as recently reported by Webb and Colvin (107). The lipid is probably recycled since it does not accumulate in the medium. The microfibrils so excreted become matted together with a gummy polysaccharide into a sort of textile.

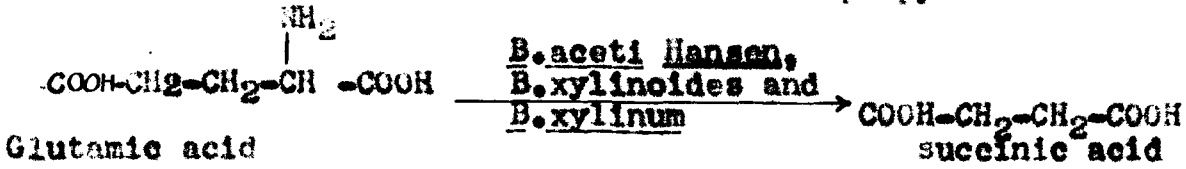
The non-cellulosic polysaccharide which is being synthesised by A.Capsulatum, A.viscosum, and A.turbidans etc. is in the form of capsules which cause ropiness in beer. Later, the polysaccharide was identified as dextran and Mehre et al. (108) are to be credited for the confirmation of this conclusion. They proposed that dextrin acts as a primer for accepting glucosyl units, analogous to maltodextrins which acted as primers in amylose synthesis by phosphorylase. The best substrate for dextran was amyloheptaose and the synthesis was brought about by dextran-dextrinase enzyme. The smallest active primer was presumably amylotetraose. A_n amylose type of polysaccharide has also been reported (109) to be formed by A.pasteurianum in beer containing fructose. Tosic and Walker (17) reported the production of similar type of polysaccharide by A.acidummucosum on malt extract media.

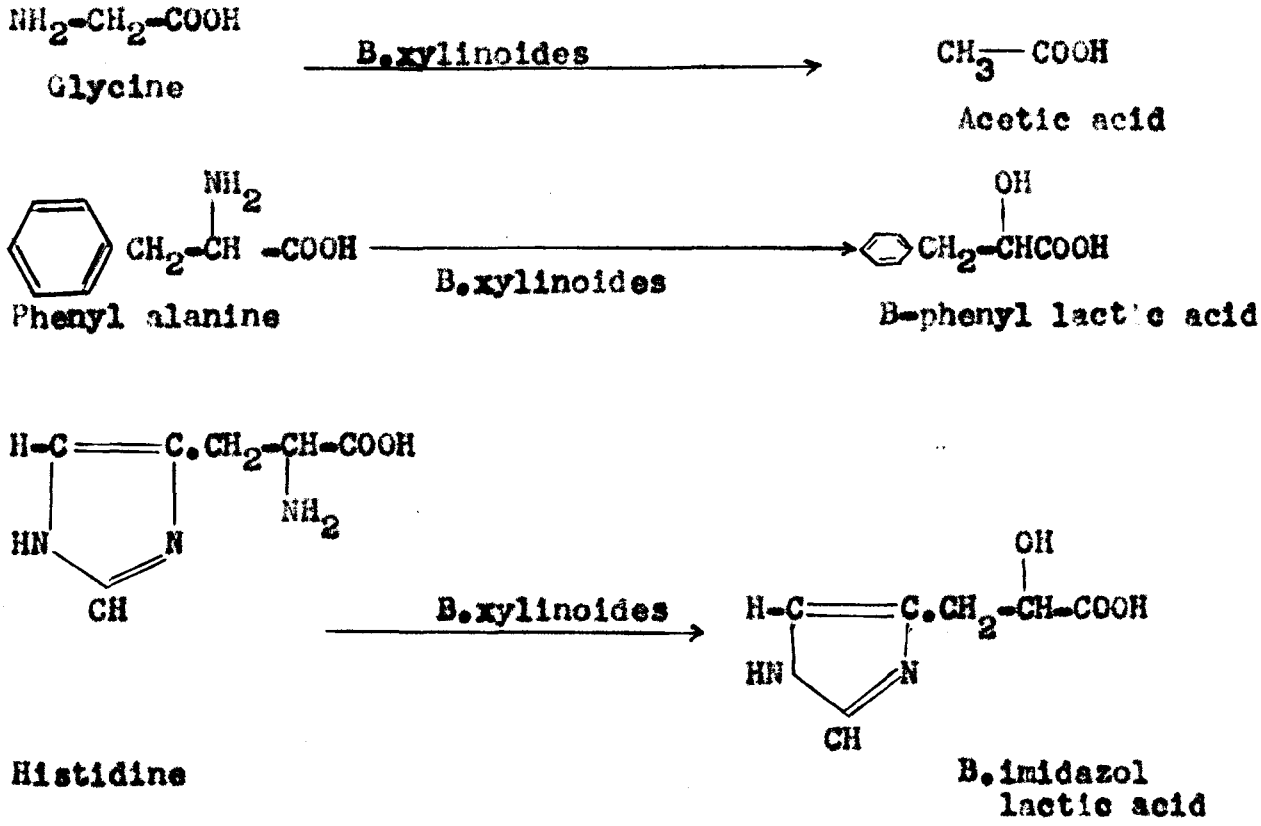
Amino acid Metabolism

The literature cited so far indicates that no attention has yet been directed towards studies on the amino acid metabolism of Acetic acid bacteria. About 38 years ago (1928), Miyaji (110), a Japanese worker, reported that some Acetobacter strains deaminated hydrolytically a number of amino acids. This was done by inclusion of the respective amino acids in growth medium and isolation of the decomposition product after three months incubation. The products obtained by various strains were as follows:









But this gave no idea as to whether the products were the cause of primary degradation of amino acids or were obtained as bye products. After 20 years, Stokes and Alma Larsen (111) reported that resting cell suspension of A.suboxydans deaminated various amino acids oxidatively but the products of deamination were not identified.

CHAPTER II

NITROGEN METABOLISM OF AMINO ACIDS BY BACTERIA

Amino acids which are the source of nitrogen for cell growth and multiplication and a source of energy of living organisms are the building stone of all kinds of proteins which are the chief organic components of cellular structure and organization. The metabolic fate of these amino acids can be treated under two heads: (1) Anabolic changes concerned with the synthesis of proteins and other biologically active compounds like vitamins, hormones, purine, pyrimidine, porphyrin etc; (2) Catabolic changes related to the decomposition of amino acids not so required. The present review is related to the latter type.

Several routes of amino acid degradation by bacteria are known; in general, however, the chemical reaction involved may be allocated to one of the four following groups:

I) Deamination or removal of alpha amino group

II) Decarboxylation or removal of carboxyl group

III) Deamination accompanied by decarboxylation

IV) Break down of amino acids into smaller units by routes other than (I) and (II)

In the pages that follow, deamination has been described in

greater detail while the remaining three modes of break down have been discussed only briefly.

(I) Deamination

Amino acid deamination is brought about by removal of NH_2 group and results in the production of ammonia. When ammonia is released by removal of N of amide group of amino acid amides, the process is called deamidation rather than deamination. There is, however, a third mechanism also in which alpha nitrogen of the amino acid, eliminated either by deamination or deamidation, does not appear in the form of free ammonia but is transferred directly in a transamination reaction. The amino group, therefore, is removed by either of the three following processes:

(1) Deamination

(2) Deamidation

(3) Transamination

It has been shown that these three types of reactions play an important role in the metabolism of all the protein amino acids.

(1) Deamination. The enzymes involved in the deamination are known as deaminases and have been reported to occur in several micro-organisms (112, 113, 114, 115). Since 1940, a lot of work on amino acid deamination has been done by several workers. Deamination may be brought about in a variety of ways, each resulting in a different product.

(a) <u>Oxidative deamination to give a keto acid</u>

$$RCH\ NH_2\ COOH + 1/2\ O_2 = NH_3 + R\text{-}CO\text{-}COOH$$

(b) <u>Non-oxidative deamination</u> to give acids other than keto acids.

I) Desaturation deamination to give an unsaturated fatty acid.

$$RCH_2\text{-}CH\text{-}NH_2.COOH = NH_3 + RCH{:}CH.COOH$$

II) Hydrolytic deamination to give a hydroxy acid

$$RCH\text{-}NH_2\ COOH + H_2O = RCH\text{-}OH\text{-}COOH + NH_3$$

III) Reductive deamination to give a saturated fatty acid

$$RCH\ NH_2\ COOH + 2H = RCH_2.\ COOH + NH_3$$

IV) Mutual oxidation-reduction between pairs of amino acids

$$RCH\ NH_2\ COOH + XCH\ NH_2\ COOH + H_2O = 2NH_3 + RCO.COOH + XCH_2\ COOH$$

(a) <u>Oxidative deamination</u>

Oxidation of amino acids by mammalian tissue has been shown to occur as long ago as 1905 by Knoop and Neubaur, but Oxidative mechanism could be known only after the most decisive contribution made by Krebs (116,117,118,119,120) in this field. Gale (121) showed that oxidative deamination of amino acid involves the production of ammonia and keto acid with an equivalent oxygen uptake if the reaction is stopped at the keto acid stage. According to Gale and Stephenson (122),

numerous amino acids were aerobically deaminated by cell suspensions of E-coli. Glyoxalic 2,4 dinitrophenyl hydrazone (123) was isolated as the oxidative deamination product of glycine by cells of E.coli, Proteus, Vulgaris, Pseudomonas flourescens and Bacillus mycoides only under aerobic conditions. Adler and coworkers (124) extracted an enzyme from E-coli which oxidized L-glutamic acid. Haemophilus parainfluenzae (125) was found to deaminate both the aspartic and glutamic acids. Decomposition of L-phenyl alanine, L-tyrosine and L-leucine by Proteus vulgaris was shown to be oxidative (126). Of 34 strains of Bact. denitrificans, 33 have/found t deaminate L-phenyl alanine and L-histidine to give the respective ketoacids (127). B.Nisman and G.vinet (128) reported that Clostridium sporogenes deaminated, with oxygen uptake, alanine, leucine, nor leucine, isoleucine, Phenyl alanine, methionine, valine, cysteine, threonine and serine to give the respective keto acids. L-alanine is oxidatively deaminated by bacterial alanine dehydrogenase which is found to be NAD dependent (129). The same has also been reported in Bacillus subtilis which deaminated alpha-amino butyric acid, serine, vinylalanine, valine, and nor leucine in addition to alanine in decreasing order (130). Recently oxidative deamination of D and L-alanine by cell free extract of Rhodopseudomonas spheriodes has been reported. Pyruvic acid was isolated and the deaminase had an

obligatory requirement for ferricyanide (131).

Enzyme systems in oxidative deamination of amino acids.

Though oxidative behaviour of cells towards amino acids is several decades old but the enzyme systems operating such mechanisms could be known only after 1932 with the opening of a new era in the field of Biochemistry by Warburg with the introduction of his new techniques in this field.

The general oxidative deamination reaction as postulated by Knoop (132) and Neubauer (133) is given as follows. It is the summation of several consecutive reactions as would be seen later.

$$RCH\,NH_2 - COOH + 1/2\,O_2 = R-\overset{O}{\overset{\|}{C}}-COOH + NH_3$$

The literature cited so far indicates that oxidation of amino acids by micro organisms, muscles, and tissues is catalysed by enzymes called amino acid oxidases (134, 135) which differ in their substrate specificity, origin and properties and have been divided into three classes.

1) D-Amino acid oxidases
2) L-Amino acid oxidases and
3) Specific amino acid oxidases.

These oxidases are conjugated proteins and the thing common to all these oxidases is that their prosthetic group is either FAD or FMN (134, 136, 137, 138), except in the oxidases of

Bacteria (135) where the nature of prothetic group could not be known despite numerous efforts made in this direction. The conclusions drawn from the observations of numerous workers (139) indicate that of these oxidases D-and L-amino acid oxidases are distinguished by their sensitivity towards certain reagents. The latter (118, 135) is inhibited by HCN, and octyl alcohol, being associated with tissue particles is insoluble, while the former is soluble and insensitive to these reagents. L and D- amino acid oxidase as their names indicate will deaminate only L and D-amino acid respectively except glutamic acid, glycine and aspartic acid which are oxidized by their specific oxidases. Glutamic oxidase is NAD (140) or NADP (124,141,142) dependent while glycine oxidase (143) and aspartic acid oxidase (144) are flavoproteins, however, recently glutamic acid oxidase (145) has been reported to oxidise other amino acids than glutamic acid alone. Similarly L-alanine is oxidized by a NAD or NADP dependent enzyme most probably glutamic acid oxidase (146).

Mechanism of Action

Whether oxidative deamination is brought by L-amino acid oxidase, D-amino acid oxidase or specific oxidase the deamination to a keto acid may be described by equations 1 through 4.

$$(1)\quad NH_2 - \overset{R}{\underset{|}{CH}} - COOH + \text{Flavin} = NH = \overset{R}{\underset{|}{C}} - COOH + \text{Flavin } H_2$$

(2) $NH = C(R) - COOH + H_2O = O = C(R) - COOH + NH_3$

(3) Flavin $H_2 + O_2 = H_2O_2 +$ Flavin

(4) $H_2O_2 \longrightarrow = H_2O + 1/2\ O_2$

$$NH_2 - CH(R) - COOH + 1/2\ O_2 = O = C(R) - COOH + NH_3$$

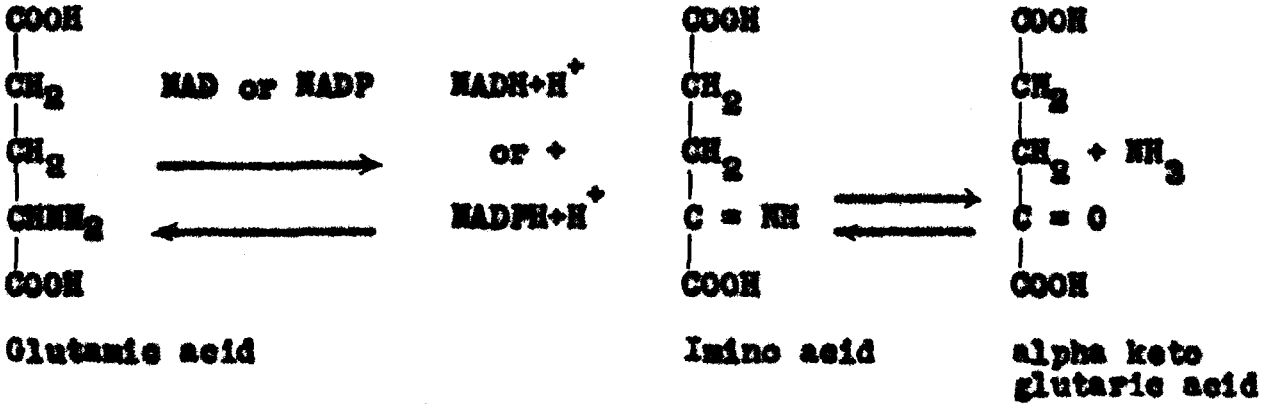
Reaction No.1, the primary step, is catalyzed by oxidase (aerobic dehydrogenase) to form an imino acid which in its turn is hydrolysed spontaneously (non enzymically)to give keto acid and NH_3 (reaction No.2). The fate of 2H produced is shown by reactions 3 and 4. In some instances as reported by G. Taborsky (147), however, the first step may yield an alpha-beta unsaturated amino acid which on subsequent hydrolysis produces ammonia and keto acid:

$$NH_2-CH(CH_2R)-COOH \xrightarrow{-2H} NH_2 - C(=CHR)-COOH \xrightarrow{H_2O} O = C(=CH_2R) - COOH + NH_3.$$

But the possibility is that neither of the two intermediate acids are formed in free state and hydrolysis occurs while the dehydrogenated substrate is attached to the flavoprotein (148).

The systems where flavoprotein is not involved, the hydrogen acceptor is other coenzyme than flavin, as is seen

in case of glutamic acid oxidase below. Glutamic acid oxidase has NAD or NADP as its coenzyme (134, 140, 141, 142)

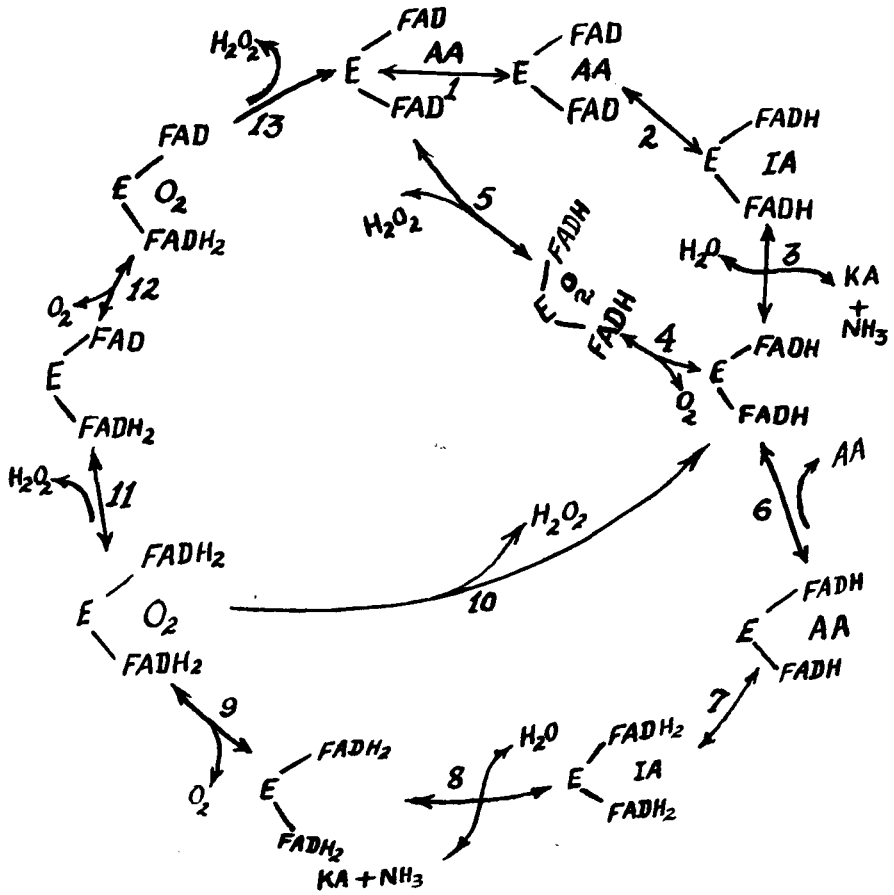


Glutamic acid — Imino acid — alpha keto glutaric acid

Sources of amino acid oxidase.

L-and D-amino acid oxidases are widely distributed in mammalian liver and Kidney (118, 134, 149, 150), in snake venom (151), bacteria (135,152) and molds (138,153,154,155). Glutamic oxidase is found in many bacteria (134, 141), plants (156, 157) and animal tissues (140, 158). Glutamic oxidase of beef liver has been crystallized and found to contain zinc (139). Glycine oxidase is found in the liver or kidney of mammal (143). Liver and kidney of rabbit has been reported to contain aspartic acid oxidase (160). It is also present in bacteria (*E.coli*) (161). Both D-and L-amino acid oxidases of sheep kidney and of the liver and kidney of rat have been isolated and purified (118,134,149,150). Singer (136) isolated from snake venom L-amino acid oxidase which is most active of

all the amino acid oxidases studied so far, having its turn over number 3100 as compared to 2000 and 6 for mammalian D-and L-amino oxidase respectively. Recently (1960-1961) Wellner and Meister (162,163) have obtained a crystalline L-amino acid oxidase from crotalus admanteus (snake), one mole of this contains 2 FAD. They also proposed a tentative scheme of its mechanism as given in Fig.1 and described below:



(FIG. 1)

IA = alpha-imino acid, A.A = alpha amino acid, K.A = Keto acid, FAD = Flavin adenindinucleotide.

Tentative scheme for the mechanism of L-amino acid oxidase

(D.Wellner and A.Meister J.Biol. Chem. 236, No.8, 2363 (1961)

The amino acid forms a complex with L-oxidase donating 2 hydrogen atoms, one to each FAD and yielding dehydroenzyme, and an imino acid which gets hydrolyzed to NH_3 and a keto acid (Fig.1, step.1,2 and 3). This half reduced enzyme is reoxidised by oxygen (step 4 and 5). In absence of oxygen, a second amino acid mole reacts with half reduced enzyme to yield a fully reduced enzyme (steps,6,7 and 8) which reacts slowly with oxygen (steps 9-10). It is also possible that oxygen may react with the fully reduced enzyme by removing 2H from the same FAD (step 11) to yield an enzyme containing one reduced and one oxidised FAD. Further more it reacts with oxygen to yield fully oxidized enzyme. (step 12). Burton (138) reported both kinds of oxidases in the extracts of 17 strains of Neurospora which deaminated 33 amino acids.

L-amino acid oxidase activity has also been reported in the extract of Proteus vulgaris (136). This enzyme was found to be inhibited with octyl alcohol and cyanide (NaCN). The activity was associated more to the particulate fraction than to the cell free state. Nature of coenzyme could not be known. Fresh cell suspension deaminated (oxidized) 22 different amino acids but cell free extract or aged suspension deaminated only eleven.

An equivalent enzyme has also been reported (135) in Aerobacter aerogenes and Pseudomonas pyocyaneus. Oxidation of amino acids by Clostridium sporogenes and Cl. saccharo

butyricum and the properties of enzyme involved have been described by Rosenberg and Nisman (152). Many bacteria viz. Proteus, Pseudomonas, Bacterium cadavaris, E-coli (135,164,165, 166) are known to metabolise both isomerides of amino acids. But the concerning enzyme could not thoroughly be studied. Stumph and Green (167) obtained a D-amino acid oxidase preparation from Proteus morganii. The enzyme was asecociated with insoluble cell particles. Amino acids were oxidized by this enzyme according to the general equation of amino acid oxidation. Nature of coenzyme could not be known.

(b). Non-oxidative deamination.

Non-oxidative deamination is brought about by large number of microorganims (168,169,170,171) and plant tissues. It is beyond the scope of the present review to go in the details of each systems since only few enzymes concerning these systems have been studied in cell free state. However, a short account /of its various types already enumerated will be given here.

(I). Desaturation deamination:

The system has been reviewed by Arkama and Virtanen (172). The deamination products are unsaturated fatty acid and ammonia. For example, the deamination of L-aspartic acid to give fumaric acid by cell free enzyme of Pseudomonas fluorescens as proved by virtanen and Tarnanen (171) was

brought about by this system. The reaction is catalyzed by aspartase (116,169,171). Aspartase activity of E-coli was greatly accelerated by adenosine or inosine as reported by Gale (173). Bivalent metal ions (Mg^{++}, Mn^{++}, Co^{++}) are reported to stimulate the activity of this enzyme in Proteus (x19) (174); and the inactivation caused by dialysis of aspartase of propionic acid bacteria was reversed by other metal ions (Ca^{++}, Mn^{++}, Ba^{++}, Pb^{++}, Sr^{++}, Co^{++}, Ni^{++}, Fe^{++} and Zn^{++}) (175). Studies after considerable purification of the enzyme from the extract of Bacterium cadavaris has been done by Williams and McIntyre (176). The reaction seems to be reversible as high yield of aspartic acid was found by incubating fumaric acid, ammonia and cells of various bacteria (Serratia marcescens, E.coli and Bact.succinii) (177). Another enzyme analogous to aspartase is histidase or histidine deaminase (178) only specific for deamination of histidine to give ammonia and urocanic acid (unsaturated acid). This is present in bacteria (170,179,180). Purified enzyme has been prepared both from liver (181,182,183) and from Pseudomonas fluorescens (184).

Histidase (185) is activated by thiol compounds like glutathione and also by sulphite. Histidase of a soil bacteria (186) has recently (1953) been isolated and found to be inhibited by Mn^{++}, Fe^{++}, Ba^{++}, Ag^{+}, Pb^{++}, KCN and 8-hydroxy quinoline. Dialysed inactivated extract was reactivated by Mg^{++}, Hg^{++} and Zn^{++}.

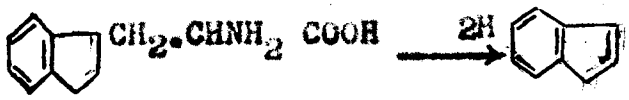
II. Hydrolytic deamination.

There have been several examples of hydroxy acids being formed during the deamination of various amino acids by bacteria (187-190,110). Virtanen and Erkama (191) reported that aspartic acid is deaminated irriversibly to give malic acid and ammonia. Fumaric acid was not the intermediate in the formation of malic acid from aspartic acid as the bacterial enzyme preparation was done in such a way so as to inhibit the fumarase activity. Therefore, the enzyme system for hydrolytic deamination must be different from other deaminase systems. Miyaji (110) isolated the corresponding hydroxy acid by the decomposition of various amino acids from the old culture fluid of acetic acid bacteria. But they did not confirm whether hydroxy acids were the direct out come of amino acid deamination or were produced from some other intermediate. Most recently hydrolytic degradation of arginine and guanidine derivatives has been reported by Jean Roche (192).

III. Reductive deamination.

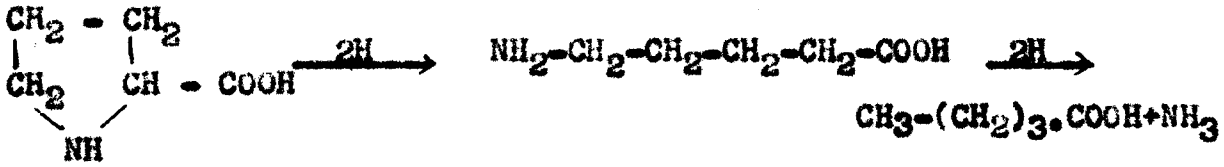
The microbial production of saturated fatty acids from corresponding amino acids has been reported by various authors (194,195,196). Woolf and Cook (193) isolated succinic acid as the deamination product of aspartic acid by washed cell suspension of 11 representative of aerobic,

anaerobic and facultative anaerobic bacteria. Later Woods (197) proved that deamination of tryptophan by E.coli occurs as follows:



Tryptophan Indole propionic acid.

Recently (198) glycine is reported to be reductively deaminated by Clostridium stick landii to give acetic acid and ammonia. Evidence (199) is also presented for reductive cleavage of proline by rumen microorganisms in vitro to form valeric acid



IV. Mutual oxidation and reduction between pairs of amino acids.

In this type of deamination strict anaerobic micro-organism like Clostridium sporogenes and Clostridium botulinum activate certain amino acids as hydrogen donators and others as hydrogen acceptor resulting a coupling reaction between them (200,201). This reaction is known as "Stickland reaction" as it was first studied by Stickland (200,201). For example - alanine and glycine when incubated together with the cells of Clostridium sporogenas (202) the over all reaction is as follows:

$CH_3\ CH\ NH_2COOH + 2\ NH_2\ CH_2\ COOH + 2H2O \longrightarrow 3\ CH_3COOH + 3NH_3 + CO2$

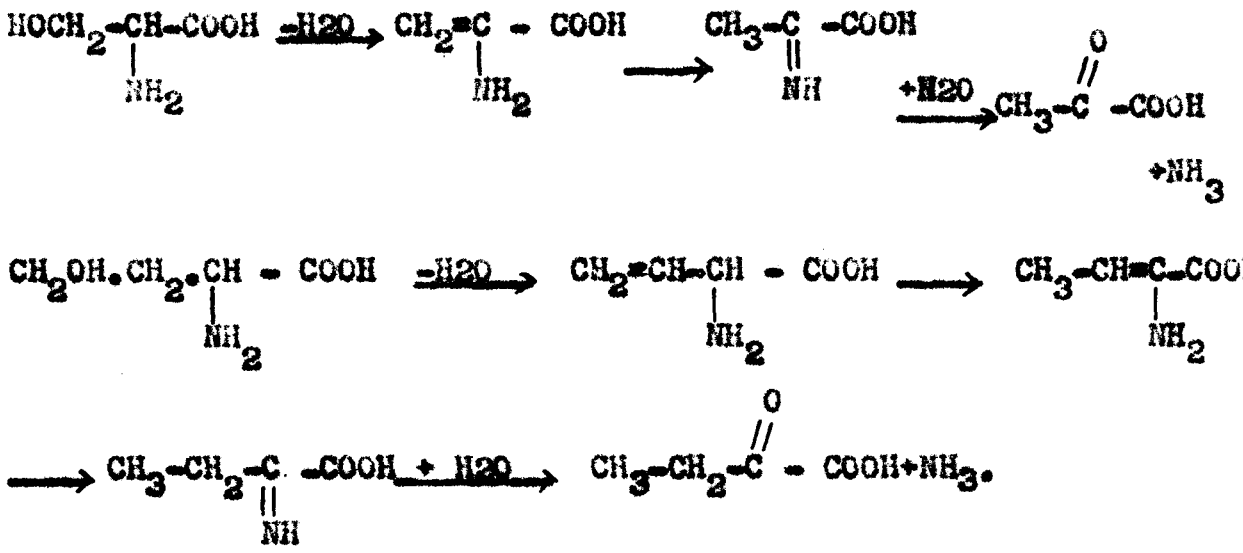
The mechanism of this reaction is fully studied (203) and it is found that one mole of acetic acid is produced by oxidative decarboxylation of pyruvic acid and 2 moles by glycine reduction. Preparation of Cl. aurogenes also contains a "proline "reductase" system which converts L-proline to δ- amino valeric acid when proline is coupled to the oxidative deamination of alanine. The over all reaction is as follows:

L-Alanine + 2L-Proline $\xrightarrow{2H2O}$ Acetic acid + 2 δ-Amino Valeric acid $+NH_3+CO_2$

Special type of non-oxidative deamination.

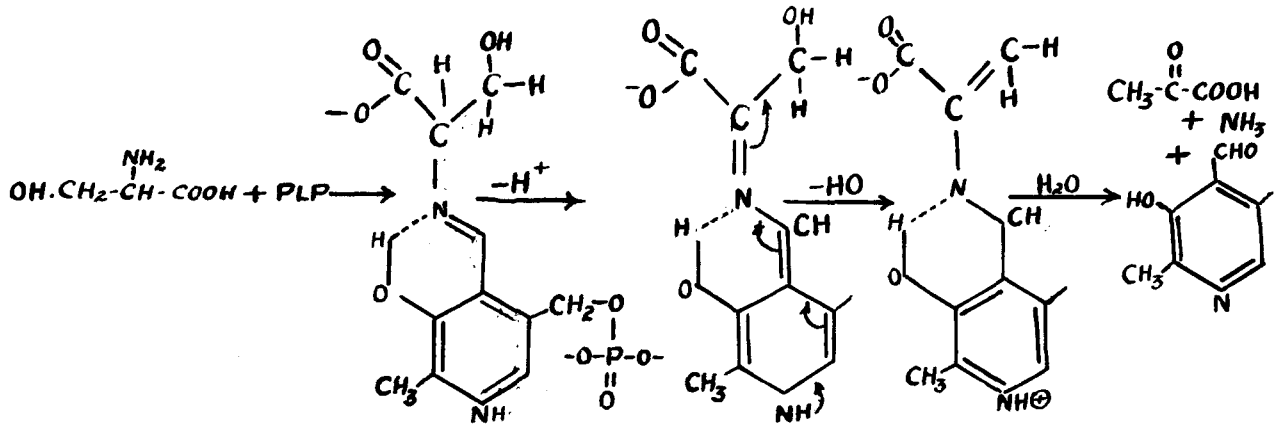
Though keto acids are the deamination product of certain amino acids like serine, homoserine, threonine, cysteine, and tryptophan, but since enzymes concerning such deamination are neither amino acid oxidases nor any specific oxidase and therefore, such types of deamination have been kept under an especial type of non-oxidative deamination and are brought about by enzymes called dehydrases in case of hydroxy amino acids, desulfhydrases in SH-containing amino acids and tryptophanase in case of tryptophan deamination: serine, threonine and homoserine dehydrases are widely distributed in bacteria (204-210), molds, (211-214) and in animal tissues (215-217). The enzymes bring about the primary dehydration or desulfuration of hydroxy amino acids and

sulfhydryl containing amino acids respectively to give , unsaturated amino acid which on subsequent spontaneous deamination yields the respective keto acids. The sequence of reactions are as follows:



It is noted that all these enzymes are pyridoxal phosphate dependent except L-threorine and L-serine (substrate of single enzyme) dehydrase of E-coli reported by Woods and Gunsalus (206) which was shown to require AMP and GSH for reactivation. And the mediation of PLP in this process takes place through a

schiff base formation with hydroxy amino acids as proposed by Metzler and Snell (218) below:



Serine deaminase is present in many bacteria (205,207,210,208) (E-coli, Pseudomonas aeruginosa, Proteus ox-19, Clostridium welchii) and in Neurospora (205,210). Two distinct enzymes one active on D - and other on L - form of serine have been reported in E-coli (205,207,219) and in Neurospora (212,220). Tryptophanase which breaks the trytophan into indole, pyruvic acid and ammonia is also present in many bacteria. (221,222). Cysteine and homocysteine dehydrases have been reported in many bacteria (223-227). A new PLP dependent enzyme called L-methionine demercapto deaminase which catalyses the break down of L-methionine to CH_3SH, NH_3 and $CH_3-CH_2-C^{\alpha}-COOH$ has been reported in clostridium sporogenes (228).

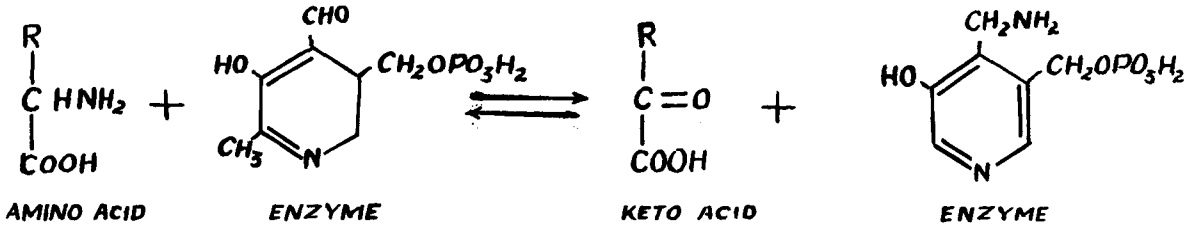
(2). Deamidation.

By this process amino acid amides are deamidated by enzyme deamidase to give ammonia and amino acid. Glutaminase and asparginase (229,233) are most important deamidases and have been reported in many microorganisms, plants and animal tissues. Glutamine and asparagine are their respective substrates. Deamidation has been reviewed by various authors (229,234).

(3). Trans amination

Transamination a general metabolic reaction for both the synthesis and degradation of L-amino acids, and first described by Braunstein and Kritzmann (235) is brought about by transaminases, which are widely distributed in microorganisms animal tissues and plants (236-238). D-amino acid transaminases are also reported to exist in Bacillus anthricus (239,240) and Rhodospirillum rubrum (241). Bacillus subtilis possesses transaminases specific for both D- and L-amino acids. Transamination occurs by exchange of amino group of amino acid to a keto acid producing thereby corresponding keto and amino acids. The most active and widely distributed of all the transaminases are the glutamic-oxalacetic transaminase (242-247). It is not limited between alpha keto acid and amino acids, but it also acts between adenine and keto acid (248) and amino acid amides and keto acid (249). Pyridoxal phosphate is the prosthetic group of all

transaminases so far studied (250,251) and plays the role of amino group acceptor and donor in accordance with the following formulation (252,253):



Factors influencing Bacterial Deamination

"Age of culture" The deaminase activity of bacterial cells varies with the age of culture from which the cells have been harvested (254). Gale and Stephenson (206) showed that serine deaminase activity was from 200 for 6 hours old culture to 1100 for a 12 hours old culture. After 12 hours growth the activity falls down. And this fall in activity as reported by later workers was due to the metabolic changes in the properties of the growth medium brought about by the cells (173). Hence to secure a true picture of enzyme make up of the organism it is necessary to examine the activity of cells after various stages of growth.

"Aerobiosis and Anaerobiosis" Gale and Stephenson (173,206,122) reported that oxidative deaminases of E.coli are best formed by growing

the culture under aerobic condition. Aerobiosis and anaerobiosis of incubation mixture (consisting of washed cells buffer and amino acid) were also found to be much effective in deamination of amino acids. Serine deamination by Lactobacillus casii (255) was stimulated by an aerobic condition. However, in Bacterium coli, Pseudomonas pyocyanea and Proteus ox19, the deamination of serine was increased by aerobic condition. Alanine was not deaminated to any extent under anaerobic condition but ammonia production was abruptly increased by shaking the incubation mixture in presence of air as reported by Chargaff and Sprinson (204) in the above mentioned species.

"Effect of carbohydrate during growth on deamination"

It has been a general finding that all amino acid deaminases (173,122,206,255,256,257) are inhibited by growing the culture in presence of fermentable sugar. And this effect has been reported as early as 1912-1922 by Kendal and his coworkers. They concluded that "sparing" action of glucose on deamination of protein was due to the fact that the carbohydrate provided a good source of carbon and energy for the organism to grow with the result that it decomposed less nitrogenous material (proteins and amino acids). Raistrick and Clark (258) gave another explanation that ammonia produced

during deamination in growth medium is being utilized for cell production in presence of carbohydrate and hence ammonia production could not be detected in such cases. A tryptophanase obtained from culture of E.coli grown in presence of glucose was also found to be inactive as reported by Happold and Hoyle (259,260). Later Gale and Stephenson (122) studied the effect of glucose in growth medium not by incubating the respective amino acids in growth medium but by incubating them with the completely washed cells so obtained, outside the growth medium, and showed that even in this case the deaminating abilities of such cells were either completely disappeared or it was vanished to a greater extent. And finally they concluded that glucose had no action upon the actual deaminating process but affected the enzyme make up of the cell during growth. They after extending their work with other amino acids (206,173) suggested that the effect was neither due to anaerobiosis nor due to fall of pH of growth medium as bubbling the medium with oxygen and maintaining the pH of the medium near neutrality did not alter the effect. Recently (1960) it has been reported that addition of glucose in the growing culture of E.coli at any time prior to the initiation of enzyme synthesis prevents completely the formation of a tryptophan deaminase (261). The effect of glucose varied with the kind of amino acid incubated (262),

the nature of sugar used (222), and the amount of sugar added upto a certain limit. It was reported that activity decreased with increasing amount of glucose upto a certain limit (255). In contrast to this general finding it has also been reported (262) that a promoting effect was found for L-histidine deaminase of Proteus vulgaris. And this was a new type of deaminase reported and was named as "oxydodeaminase". Mechanism of this inhibitory effect of glucose is still not clear. However, two observations (261), the absence of a demonstrable effect of the carbohydrate on tryptophane permease, and the marked stimulation of tryptophan synthetase by concentrations of glucose significant to inhibit tryptophanase may prove significant. This inhibitory effect of glucose is also seen in culture of an algae where deamination of glycocol was inhibited (263).

II. Decarboxylation.

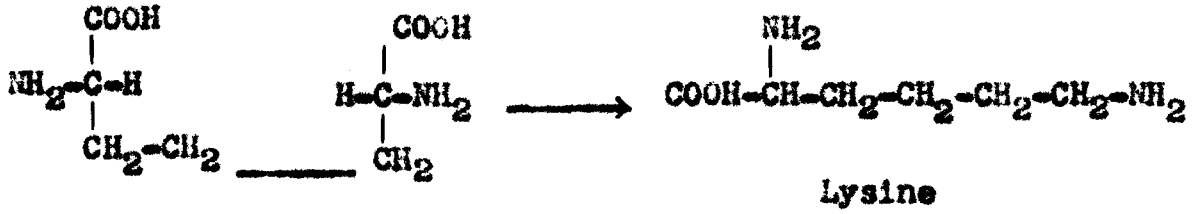
Decarboxylation is another process of amino acid break down in biological systems. It is brought about by a specific pyridoxal-dependent enzyme, known as amino acid decarboxylase. The decomposition products are CO_2 and the corresponding amines. The reaction occurs as follows:

$$R\text{-}CH\ NH_2\ COOH \xrightarrow[\text{amino acid decarboxylase}]{B_6\text{-}PO_4} RCH_2NH_2 + CO_2$$

Enzymes of this group are widely distributed in many bacteria, [E.coli (190,264,265), Proteus species (190), Pseudomonas species Salmonella species (266), Aerobacter species (266), Clostridium species (267,266) Streptococcus species (268)] , animal (269) and in plant cells (270). The first study of amino acid decarboxylase was done by Gale (265) who reported that of 14 strains of E.coli investigated;12, decarboxylated arginine to agmatine; 12, histidine to histamine; 13, lysine to cadaverine; 12, ornithine to putriscine; and 9, glutamic acid to α -amino butyric acid. He after extending his studies (271) with other organisms, demonstrated that the production of decarboxylases is very much influenced by the environmental conditions, such as pH and temperature of the growing medium. The culture grown at 20°-25°C contained more active enzymes than grown at 37°C. However, glutamic decarboxylase of Proteus vulgaris (267) was less thermolabile, and histidine decarboxylase (267) was formed better at 37°C than at lower temperature. It is a general finding of all amino acid decarboxylases, that they are formed in response to acid growth condition. The pH optimum is below 5.5 in large number of cases and below 4.5 in most cases. Decarboxylase activities of Clostridium welchii and E.coli have been studied in great detailed, and it was found that glutamic acid decarboxylase of these organisms was similar in properties; but histidine decarboxylase of Clostridium welchii had its optimum pH from 2.5 to 3 and in E.coli it was 4. And this enzyme of E.coli was completely inactivated at pH 3.5.

And the explanation of this variation in pH was given that histidine decarboxylase had greater resistance to denature of the proteins in Clostridium welchii over those of coliform organisms. Since histidine decarboxylase of Cl.welchii required more acid condition for its synthesis, therefore, it was produced late in glucose broth medium when the pH had considerably fallen due to excessive acid production. This finding also indicated that decarboxylase activity of cell suspension towards a particular amino acid varies with the age of culture. The young cells show little activity which increases with the age of the culture and reaches to maximum as growth ceases and after which much acid has been produced (267,271).

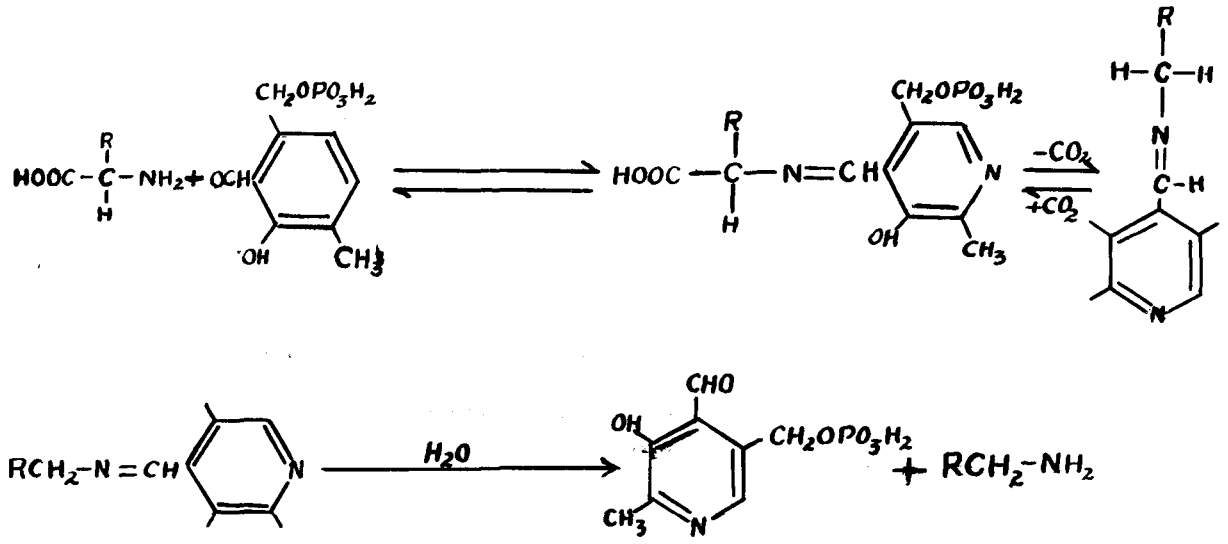
The decarboxylases are adaptive enzymes as they are formed in large quantities by growing the cells in presence of a specific substrate. However, diamino pimelic acid decarboxylase (272-274) is a constitutive enzyme and brings about the decarboxylation of meso-α, ϵ -diamino pimelic acid to give lysine as follows:



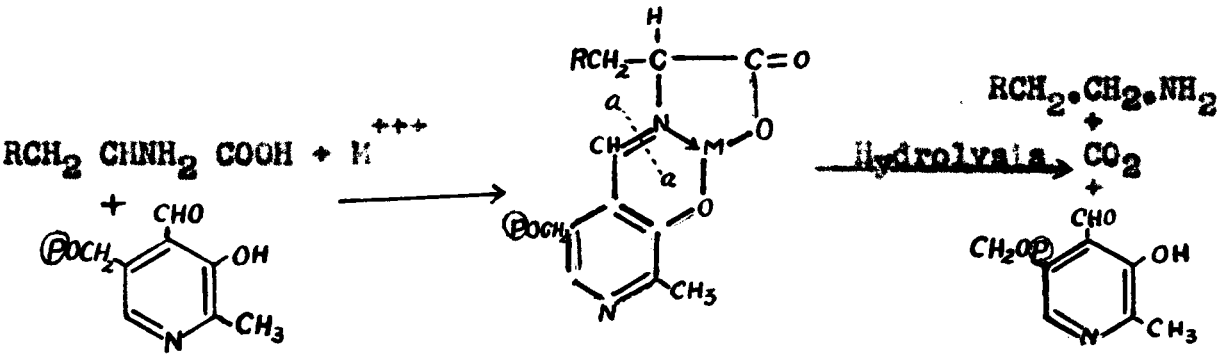
Meso-α, ϵ -diamino pimelic acid

Amino acid decarboxylases are generally specific towards their substrate and only L-form of a single amino acid is attacked by a specific decarboxylase. However, L-tyrosine decarboxylase of Streptococcus fecalis also attacks 3,4 -dihydroxy-L-phenyl alanine as well as tyrosine, it also decarboxylates L-phenyl-alanine but at a very slow rate. Likewise, L-leucine and L-valine have also been decarboxylated by a single enzyme (275) in case of Proteus vulgaris.

As reported earlier all the enzymes of this group are found to have pyridoxal phosphate as the coenzyme; histidine decarboxylase (276) requires, in addition, a metal ion (Fe^{++} or Al^{+++}). Recently glutamic acid decarboxylase of E.coli (264) has been prepared in 90% purity and is shown to require two pyridoxal phosphate residue per mole of apoenzyme. The postulated mechanism of the decarboxylation reaction according to Mendeles, Koppilaman and Hanke (277) is as follows:



Later Snell (278) gave an explanation for this mechanism that amino acid combines with a trivalent metal and pyridoxal phosphate to give a chelate ring system, which breaks up along the line a...a to yield amine, and CO_2 as follows:



Although amino acid decarboxylases are widely distributed in living organisms and take part in synthetic processes, much still remains to be learnt their role in amino acid metabolism.

III. Deamination accompanied by Decarboxylation.

Certain cases have earlier (1905-1912) (121) been reported, where deamination, and decarboxylation of amino acid occur simultaneously by microorganisms to give the following one ofthe chemical reactions:

$$RCH.NH_2\ COOH + H_2O \longrightarrow RCH_2\ OH + NH_3 + CO_2$$

$$RCH\ NH_2\ COOH + 2H \longrightarrow RCH_3 + NH_3 + CO_2$$

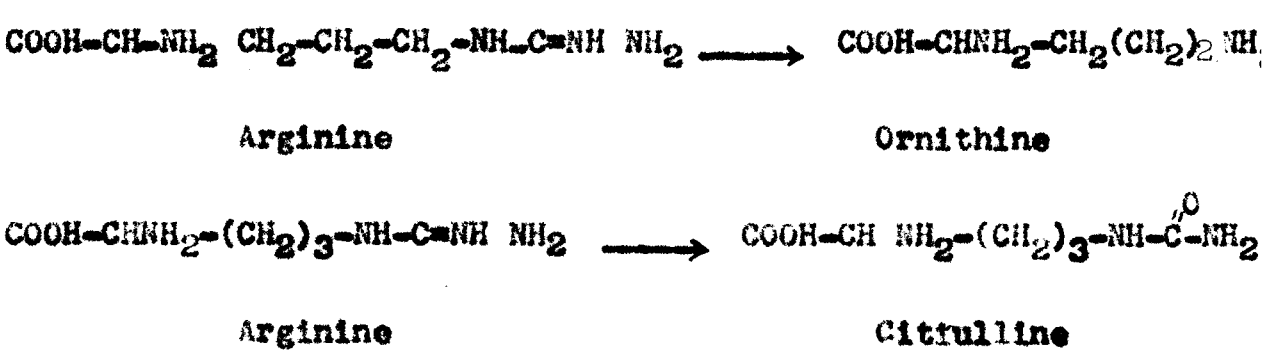
$$RCH\ NH_2\ COOH + H_2O + 2O \longrightarrow RCOOH + NH_3 + CO_2 + H_2O$$

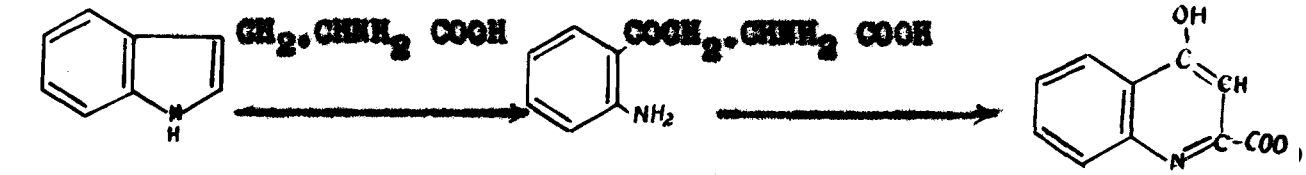
But Gale, (1[illegible],265) after studying the pH activity of glutamic acid decarboxylase and deaminase of E.coli reported that pH optima of these enzymes were not only widely separated and on opposit side of neutrality but that neither enzymes was at all active when the other was optimally active. And he suggested therefore, that both the reactions could never be occured simultaneously. And also the general pH optima of all deaminases and decarboxylases so far studied indicates that the former are active at pH 7.5-8 whereas the latter at or below pH 5. This means for deamination to occur the $-NH_3^+$ group must be undissociated and for decarboxylation reaction-COOH group must be intact. Therefore, Gale's suggestion seems quite probable that amino acids can not be degraded by both the processes simultaneously. And those cases where both the processes were referred to occur simultaneously, the experiment usually lasted over several days. And this long incubation brought considerable change in pH due to metabolic activities of the growing organisms. Thus deaminases and decarboxylases so formed at separate time under separate pH acted upon the amino acids separately rather than simultaneously to give the products of deamination and decarboxylation.

(IV). <u>Splitting of the amino acid molecule by route other than deamination or decarboxylation.</u>

A number of cases have been reported (279,280,281,268)

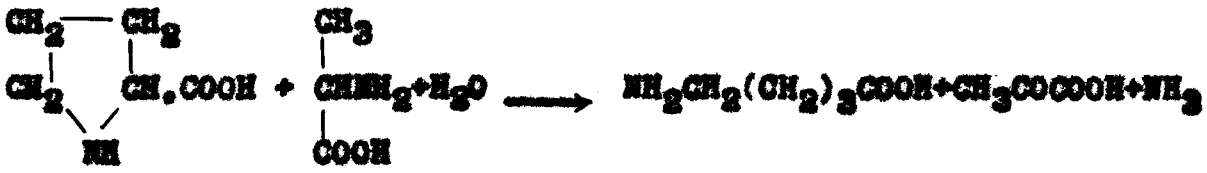
where the authors have stated that by incorporation of various individual amino acids in culture of various microorganisms the decomposition of amino acids was brought about by neither of the three main groups of attack allocated for primary degradation of amino acids. In such cases amino acids were either split into another smaller molecule of amino acid (280,281 (Arginine ⟶ ornithine) or to some other products by fission of the ring of amino acids, like tryptophan (282) and tyrosine etc. Thus arginine was reported to give ornithine by Lactobacillus acidophilus, (266) citrulline by Pseudomonas aeruginosa (283). Bacillus subtilis (284) produced kynurenic acid, kynurinine and anthranilic acid from tryptophan in presence of glycerol and aluminium phosphate. And it was suggested that kynurinine was first produced by the reductive fission of tryptophan mole followed by the disruption of kynurinine along two paths to kynurinic acid and anthranilic acid. Likewise, it was reported by Stickland (285) that L-proline was reduced in presence of L-alanine by washed cell suspension of Clostridium sporogenes to give δ -amino valeric acid. All these reactions were reported to occur as follows:





Tryptophan → Kynurenine → Kynurenic acid

Kynurenine → Anthranilic acid



L-Proline → δ amino valeric acid.

Since in all these cases, the primary degradation products were not corresponding to the decarboxylation or deamination reactions, therefore, have been kept under a separate heading.

EXPERIMENTAL

E X P E R I M E N T A L

A brief review of the methods employed:

Three general methods are usually employed in the study of bacterial metabolism:

(i) Measuring the metabolism under normal conditions of growth and reproduction.

(ii) Inclusion of specific substances to be studied in the medium and the isolation of the products after a period of incubation. This however, suffers from a disadvantage. It may yield a bye-product rather than the intermediate products.

(iii) Study of the metabolism of specific substances under conditions where other metabolic activities of the bacteria are suppressed. This method eliminates the complicating factors of growth and can be performed by using the "cell suspension" technique. By using this technique together with methylene blue, manometric and other techniques, not only an idea of the relative rates and of the quantitative nature of bacterial attack on substrates can be obtained, but also the conditions of pH and temperature under which the enzymes involved are active can be investigated. Many workers have used

this technique in studies on carbohydrate metabolism, nitrogen metabolism and intermediary metabolism and though it allows the investigation of the properties of enzymes as they exist in bacterial cells such investigation is necessarily restricted by the presence and action of other enzymes in the cells, and by the permeability of the cell membrane towards the enzyme substrate etc.

Stokes and Alma Larsen (178) studied the ability of Acetobacter suboxydans to deaminate various amino acids by using resting cell suspension. In the present study also all the experiments were performed by adopting this method.

Measurement of Deaminase activity.

In studies on deamination, the activity of the enzyme/enzymes involved can be measured in a number of ways.

(1) By measuring the oxygen uptake by Warburg manometric technique.

If we consider the oxidative deamination of alanine, we find hat 1 mole of alanine utilizes one atom of oxygen and produces one mole each of pyruvic acid and ammonia. If the reaction is stopped at the keto acid stage, the oxygen consumed will be a direct measure of deaminase activity.

(2) By measuring the amount of keto acid produced.

This depends upon the use of 2,4-dinitro-

phenylhydrazine. A one half saturated solution of this reagent in NHCl forms a characteristic hydrazone with keto acids. When excess of 2N NaOH is added to this a transitory reddish brown colour is formed which can be read on a colorimeter and compared against a standard. This has successfully been done by Snell and Metzler (207) and others. This method, however, is applicable only in cases where the keto acid formed is not further metabolized by the enzymes of the cell.

(3) Estimating the amount of ammonia produced.

This has been used by a large number of workers and is obviously the best method. The ammonia produced is distilled into an acid solution and is estimated either titrimetrically or colorimetrically. The colour produced on the addition of Nessler's reagent is measured on a colorimeter and the amount of ammonia is calculated by referring to a standard ammonium sulphate curve.

(4) Estimating the amount of the substrate left which in the case of amino acids can be done by formol titration.

(5) If a dehydrogenase system is present in deamination reactions, the hydrogen produced as a result of deamination would reduce methylene blue anaerobically and by noting the time of reduction of methylene blue under particular conditions the activity of the deaminase can be estimated.

Materials and Methods.

Studies were undertaken on sixteen species of Acetobacter. Out of these, one was obtained from the culture collection of the National Chemical Laboratory, Poona and the rest from the National Collection of Industrial bacteria (NCIB) London.

List of organisms studied:

A. suboxydans	Poona strain
A. suboxydans	NCIB - 8036
A. peroxydans	" - 8618
A. oxydans	" - 8089
A. mobile	" - 6428
A. xylinium	" - 8745
A. xylinoides	" - 4940
A. pasteurianum	" - 8856
A. kuetzingianum	" - 3924
A. acetigenum	" - 5346
A. ascendens	" - 8163
A. orleanse	" - 7215
A. viscosum	" - 6426
A. rancens	" - 8555
A. capsulatum	" - 4943
A. turbidans	" - 6424

Media.

Active stock cultures were maintained on an agar medium consisting of 0.5% yeast extract (Difco), 1% glucose (B.D.H. Analar), 2% $CaCo_3$ and 2% agar. The medium used for growing cells for inocula and for growing cells on a large scale for the preparation of cell suspensions contained 0.5% yeast extract (Difco) and 1% glucose.

Preparation of cell suspension.

A standard procedure was adopted to prepare cell suspensions so as to maintain uniformity in all the experiments performed. 1 ml of a 24 hour old culture of the organism under study was transferred aseptically to one litre flask containing 150 cc of the sterilized medium having the above composition. The medium was sterilized for 15 minutes at 15 lb pressure and had a final pH of 6.6 ± 0.2 unless otherwise stated. The flasks after inoculation were mounted on a microid flask shaker (Griffin and Tatlock) and incubated at 30°C, with constant shaking, for a period of 24-48 hours. Incubation for a period of 24-48 hours usually resulted in sufficient growth. The final pH of the culture liquid was noted and the cells were harvested by centrifugation for 15 minutes at the rate of 4000 r.p.m. under cold conditions. The cell paste so obtained was washed with ice cold physiological saline three times to wash away

the last traces of the medium. The cells were then suspended in ice cold sterile water so as to contain 12 mg dry cells per 0.5 ml of water. This was done by adjusting the turbidity of the suspension at 0.75 (optical density) on Spekker Photoelectric Absortiometer(Hilger Model H 760) using the cell of 0.25 path length and red filter of 680 mμ wave length. The cell mass of dry cells for various turbidities was determined and it was found that 12 mg dry cells per 0.5 ml of water corresponded to this optical density under the conditions described. 0.5 ml of the cell suspension so prepared was incubated with 0.5 ml of an M/20 amino acid solution, in presence of buffers of known pH in test tubes, plugged with rubber bungs, for known periods of time (usually 3 hours unless otherwise stated) in a thermostatic water bath set at 37°C, or set to a temperature at which the experiment had to be performed. At the end of the desired time, 0.5 ml of 30% trichloro acetic acid was added to each test tube to arrest the enzymic reaction and the contents of each tube were quantitatively transferred to the steam distillation chamber of a micro -k jeldahl steam distillation apparatus for the estimation of ammonia, if any, released as a result of deamination. The results are given as microrgams of NH_3 released per 12 mg of dry bacterial cells per 3 hours and were obtained by substracting the endogenous reaction from the metabolized.

In case of A-xylinum and A-xylinoides which were cellulose producing species, the cellulosic material obtained

after centrifugation and washing was thoroughly crushed in a chilled pestle and mortar. 0.5 ml of the extract (equivalent to 20-25 mg dry weight/0.5 ml in A-xylinum and 31 mg/0.5 ml in A-xylinoides) along with fine crushed material was used for incubation with buffers and the amino acids as described above.

Estimation of Ammonia by Micro-Kjeldahl method.

For the description of the apparatus, and details of the method see Practical Physiological Chemistry - 12th edition. by Hawk, other and Summerson page 820. The steam distillation chamber was cleared of any traces of contaminating ammonia by first washing it with steam and then by performing a blank distillation. The incubation mixture, from each test tube, was transferred to the steam distillation chamber and made alkaline with 3 ml solution of borax carbonate:

50 g . $Na_2B_4O_7$
10.g K_2CO_3 (anhydrous)
1 ml-1% Thymol blue

The colour of the solution finally was blue. Under these conditions, which were strictly adhered to in each estimation, the distillation was always complete if 20 ml of the distillate were collected in the receiver. The steam generated in the boiler was passed into the chamber containing the incubation

mixture transferred from each test tube, and the process continued till about 20 ml of the distillate were collected in a small receiving flask which already contained 5 ml N/20 H_2SO_4 and was arranged in such a manner that the tip of metallic outlet of the silver condenser dipped below the surface of the receiving liquid (H_2SO_4). The receiver was removed and the sides of the outlet of the condenser were rinsed down with a little distilled water into the receiver. The distillate and the washings were then transferred to a container graduated at 45 ml, and made upto the mark by rinsing.

Colorimetric procedure.

9.0 ml of the distillate were nesselerized with 1 ml Nessler's reagent prepared as given below, and the color produced was read on Spekker Photoelectric Absorticmeter (Hilger's) fitted with a tungsten lamp. The cells used were of 0.5 cm path length, and a blue pair of filters (430 mu) was used. The optical density was measured and the concentration of ammonia at this optical density was determined by reference to an already drawn standard curve against $(NH_4)_2SO_4$.

Chemicals and reagents.

Solutions of amino acids.

All the amino acids used throughout this work were obtained from B.D.H. (Analar grade) except DL-phenylalanine

which was obtained from Eastman Kodak. The solutions were prepared in distilled water except that of DL-tryptophane and l-tyrosine which were prepared in water with a few drops of alkali as they were sparingly soluble in water. The solutions of amino acids were freshly prepared for each experiment. The amino acid buffer mixture was steamed for 0.5 hour and cooled just before addition of the cells.

Nessler's reagent. (Koch modification-Nessler's Folin reagent)

A solution of potassium mercuric iodide was prepared by dissolving 22.5 gms of iodine in 20 cc of water containing 30 gms of potassium iodide. After the ingredents had dissolved completely, 30 gms of pure metallic mercury were added to the solution. The flask containing the solution was vigorously shaken, and immersed in cold water to prevent the rise of temperature. This was continued till the supernatant liquid had just lost all the yellow colour due to iodine, and became greenish yellow. The aqueous layer was decanted into a 200 cc volumetric flask and a few drops of a solution of iodine in potassium iodide prepared in a similar manner were added till it gave a faint test for free iodine on the addition of a few ccs of a cold solution of soluble starch. This is done in order to ensure the absence of mercurous compounds in the solution. In case the mercurous compounds were present, a precipitate of mercurous iodide will be obtained. The aqueous

layer is then diluted to 200 cc. A 10% solution of sodium hydroxide was then prepared and 975 cc of this solution were added to the entire solution of potassium mercuric iodide prepared above. The solutions were thoroughly mixed, and allowed to clear by standing. The reagent as here modified does not have the tendency of causing a turbid mixture when added to ammonia solutions and does not have to be added so gradually as the original Nessler - Folin reagent. It is even slightly more sensitive for the detection of ammonia than the older reagent.

Preparation of buffer solutions. (Clark & Lubs standard buffers)

Phosphate buffers were used from pH 5.8 to 8.0 and for pH values from 8.0 to 10.0, borate buffers were used. They were prepared as follows:

Phosphate buffers.

Phosphate buffers were prepared by mixing 50 cc N/10 solution of potassium dihydrogen phosphate with different volumes of N/10 NaOH solution as shown in the table below and diluted finally to 200 cc by the addition of distilled water.

KH_2PO_4 + NaOH mixtures

pH	Volume of M/10 KH_2PO_4 in cc	Volume of N/10 NaOH in cc	Diluted to
5.8	50	3.66	200 cc
6.0	50	5.64	200 cc
6.2	50	8.55	200 cc
6.4	50	12.60	200 cc
6.6	50	17.74	200 cc
6.8	50	23.60	200 cc
7.0	50	29.54	200 cc
7.2	50	34.90	200 cc
7.4	50	39.34	200 cc
7.6	50	42.74	200 cc
7.8	50	45.17	200 cc
8.0	50	46.85	200 cc

M/10 KH_2PO_4 Solution

High grade, chemically pure KH_2PO_4 was recrystallised from redistilled water at least three times. It was dried to a constant weight at 110°C to 115°C in an oven. 13.616 gms of the dry salt were dissolved in redistilled water, and diluted to 1000 cc to give a decimolar solution of potassium dihydrogen phosphate.

Borate buffers.

Boric acid was recrystallised at least twice from redistilled water and dried in thin layers between hardened filter paper until constant in weight. 6.2024 gms of the purified H_3BO_3 and 7.456 gms of purified and dried KCl (N/10)

were dissolved in redistilled water and diluted to 1000 cc. To 50 cc of this mixed solution, different volumes of N/10 NaOH solution were added, and diluted to a final volume of 200 cc to give buffers in the pH range of 8.2 to 10.0

Boric acid (N/10) + KCl (N/10) + NaOH (N/10)

pH	Volume of N/10 Boric acid + N/10 KCl solution in cc	Volume of N/10 NaOH sol in cc	Diluted to
8.2	50	5.9	200 cc
8.4	50	8.55	200 cc
8.6	50	12.60	200 cc
8.8	50	16.40	200 cc
9.0	50	21.40	200 cc
9.2	50	26.70	200 cc
9.4.	50	32.00	200 cc
9.6	50	36.85	200 cc
9.8.	50	40.30	200 cc
10.0	50	43.90	200 cc

The pH of the buffers prepared was finally checked and corrected on a Beckman Model H2 pH meter.

Trichloroacetic acid (30%).

30 gms of high grade crystalline trichlorocacetic acid (Eastman) were dissolved in distilled water and diluted to 100 cc.

EXPERIMENT NO. 1.

Testing the ability of various Acetobacter species to deaminate various amino acids.

The presence of amino acid deaminases in various species of bacteria has been reported by several workers during studies on the primary utilisation of amino acids but reports on the presence of deaminases in Acetobacter species are not available. A few amino acid deaminases have been reported in Acetobacter suboxydans (111) only but no such information in respect of other Acetobacter species is yet available. The present study was carried out with a view to investigate the ability of the various Acetobacter species to deaminate amino acids.

Preliminary experiments with the species listed in Table No. IV and twenty three amino acids as substrates were carried out using the technique described earlier. As these experiments were performed merely to look for the presence of deaminases in the various Acetobacter species, the quantitative estimation of ammonia liberated was not carried out but its presence was detected only qualitatively by means of Nessler's reagent. The relative amounts of ammonia liberated as indicated by the intensity of the yellow colour produced have been indicated by — (nil), + (traces), ++ (moderate), +++ (strong) and ++++ (very strong).

As asparagine produced a reddish brown colour with Nessler's reagent, even in the absence of the cells in the

control tubes, evidence for deamination or deamidation of asparagine was obtained by estimating colorimetrically the intensity of the colour in tubes with and without the cells.

The results as tabulated in Table IV show that with the exception of A.capsulatum, A.oxydans and A.nasturianum, all the species could deaminate some amino acid or the other. Of the various species tested, A.aceticanum (6346 NCIB) appeared to be particularly promising and was found to deaminate DL-serine, DL-nor leucine, DL-leucine, DL-alanine, DL- phenyl alanine and L-asparagine. In subsequent work, therefore, this organism was selected for detailed study.

TABLE V.

Deamination of amino acids by various Acetobacter species

Acetobacter species	Strain No.	Time of incubation of culture	Initial pH of the medium	Final pH of the medium (after growth)	Number of amino acids deaminated
A.suboxydans	9036	48 hrs.	6.6	6.9	12
A.peroxydans	8618	40 "	6.4	6.3	9
A.rancens	8555	24 "	6.6	6.1	8
A.kuetzingianum	3924	48 "	6.5	6.0	7
A.turbidans	6424	24 "	6.4	4.7	8
A.orleanse	7215	48 "	6.8	4.2	7
A.acetigenum	5346	24 "	6.6	3.6	6
A.viscosum	6426	48 "	6.8	4.9	6
A.suboxydans	Poona strain	45 "	6.7	6.4	6
A.mobile	6428	48 "	6.6	3.8	6
A.ascendens	8163	48 "	6.8	4.9	5
A.xylinum	8745	48 "	6.4	4.9	4
A.xylinoides	4940	48 "	6.4	4.4	3
A.pasteurianum	8356	24 "	6.5	4.8	None
A.capsulatum	4943	48 "	6.7	3.5	None
A.oxydans	8089	40 "	6.4	4.3	None

EXPERIMENT NO. 2.

Factors affecting the deamination of amino acids by Acetobacter aceti-ceum (5346 NCIB)

The deamination of amino acids is influenced by various physico-chemical factors and before undertaking its detailed study, it appeared necessary to find out optimum and well defined conditions for the deamination of the various amino acids by resting cell suspensions of A.aceti-ceum.

(a) Effect of pH

It is a general finding that nearly all the amino acid deaminases are found to be more active in the alkaline range. A suitable pH for the deaminases of alanine, phenyl alanine, leucine, nor leucine and serine in A.aceti-ceum was determined by taking buffers of different pH values as given in Table No. VI and measuring the amount of ammonia released per 12 mgs. (dry weight) of the bacterial cells at 37°C after a period of three hours. Borate buffers were used for pH values above 8 and it was observed that the pH optima in the case of DL-alanine and DL-nor leucine were 7 and 7.8 respectively, 7.4 in the case of DL- phenyl alanine and DL-leucine, and 8 in the case of DL-serine. The results obtained are summarized in Table No. VI.

TABLE NO. VI.

Effect of pH on the deamination of DL-α-alanine, DL-β-phenyl alanine, DL-leucine, DL-nor leucine and DL-serine by Aerobacter aerogenum.

pH (of the buffer and the amino acid solution).	DL-α-alanine	DL-β-phenyl-alanine	DL-leucine	DL-nor leucine	DL-serine
	Ammonia* released in µ gms.				
5.4	92.0	38	30.0	46	10
5.8	99.0	50	35.4	60	16
6.0	103.0	58	38.0	64	18
6.3	108.8	64	43.6	70	25
6.8	118.0	70	47.6	84	34
7.0	121.0	74	54.0	86	50
7.2	116.0	76	56.0	88	62
7.4	115.0	78	62.0	90	74
7.8	102.0	73	44.0	100	108
8.0	90.0	68	36.0	92	116
8.6	-	-	-	-	100
9.0	-	-	-	-	82
9.5	-	-	-	-	65

* NH_3 released is expressed in µ gm per 12 mg (dry weight of) bacterial cells por 3 hours at 37°C.

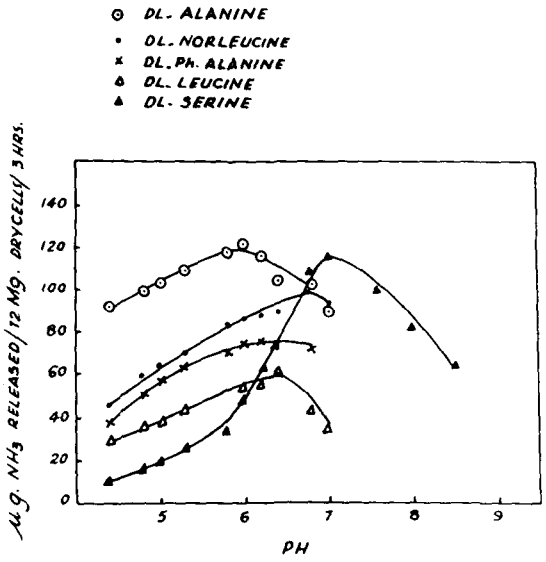


FIG. 2. EFFECT OF PH ON DEAMINATION OF AMINO ACIDS BY A-acetigenum
(REFER TABLE VI)

(b) Effect of temperature

Optimum temperature for the deamination of amino acids by bacteria usually varies from 30° to 40°C. In the present study, the optimum temperatures of all the five amino acid deaminases were found out by incubating the cell, amino acid and buffer mixture adjusted to a predetermined optimum pH, as found out earlier, at temperatures from 20° to 50°C and estimating the amount of ammonia released, in each case, under the conditions of the experiment.

The results obtained, as tabulated in Table No. VII, indicated that 40°C was the optimum temperature for alanine, phenyl alanine, leucine and nor leucine deaminases and 45°C for serine deaminase. The optimum temperature for serine deaminase in A.anatigenum appears to be rather higher than found for most of the bacterial deaminases. However, two serine deaminases of lactobacillus have also been reported to have optimum temperatures as high as 46° and 82°C at pH 8.3 and 4.0 respectively (255).

TABLE NO. VII.

Effect of temperature on deamination of DL-α-alanine, DL-β-phenylalanine, DL-Leucine, DL-nor leucine and DL-serine by Acetobacter aceticerum (6346).

Temperature	μl gas of NH_3 released per 12 mgs dry weight of bacterial cells per 3 hours at optimum pH							
	20°C	25°C	30°C	35°C	40°C	45°C	50°C	Optimum pH
Amino acids								
DL-α-alanine	42	52	76	86	130	92	-	7.00
DL-β-phenyl-alanine	10	19	34	54	76	58	-	7.4
DL-leucine	8	12	26	46	66	50	-	7.4
DL-nor-leucine	18	30	44	72	110	94	-	7.8
DL-serine	22	31	36	43.6	76	120	64	8.00

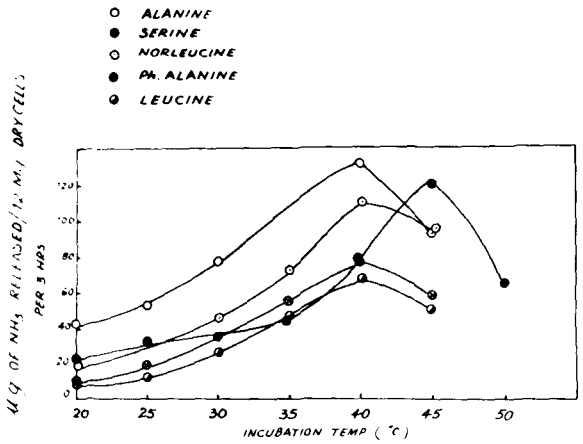


FIG 3 EFFECT OF TEMPERATURE ON DEAMINATION OF AMINO ACIDS BY A. aceti*genum* (REFER TABLE VII)

(c) Effect of shaking

Oxidative deamination of amino acids is very much favoured by shaking the mixture containing the amino acids and the bacterial cells serving as a source of the enzyme (due to an adequate supply of oxygen) in strict and most of the facultative aerobes (204).

Acetobacters are highly oxidising organisms and if the break down of amino acids by these organisms occurs by an oxidative mechanism, one would expect an increase in the rate of deamination due to an adequate supply of oxygen. This experiment was, therefore, undertaken to see if the degree of deamination showed any increase in presence of excess of oxygen supply.

Amino acids, the cell suspension and the buffers of optimum pH with respect to each of the amino acids were incubated at their respective optimum temperatures in a water bath in the usual way. Vigorous aeration was provided by shaking the flasks on a Microid Flask Shaker and controls for each set of experiments were also set up under similar conditions with the only difference that the flasks were kept stationary. The ammonia liberated corresponding to the various amino acids under the two conditions viz, with and without shaking, was estimated. The results obtained are tabulated in Table No. VIII.

It would appear that with the exception of DL-serine, where no appreciable difference was observed, in the remaining four amino acids, the amount of ammonia produced as a result of deamination was much more when the flasks were subjected to shaking than otherwise, when they were kept stationary.

TABLE NO. VIII.

Effect of shaking (adequate oxygen supply) on the rate of deamination of DL-α-alanine, DL-β-phenylalanine DL-leucine, DL-norleucine and DL-serine by Acetobacter aceti-rerum.

Amino acids	μ gms of NH_3 released per 12 mgs (dry wt. of bacterial cells) per 3 hours at 40°C and in serine at 45°C		
	with shaking	without shaking	pH
DL-α-alanine	380	118	7.00
DL-β-phenyl-alanine	120	78	7.4
DL-leucine	114	56	7.4
DL-nor-leucine	152	104	7.8
DL-serine	100	96	8.00

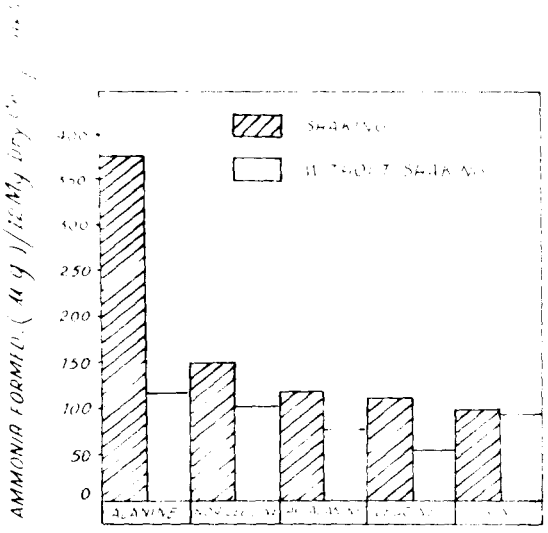


FIG 4 EFFECT OF SHAKING ON DEAMINATION OF AMINO ACIDS BY A. *acetigenum*
(REFER TABLE VIII)

(d) Effect of glucose (in the growth medium) on the growth of A.acetigenum and on its relative level of deaminase activity.

Gale and Stephenson, working with E.coli, were the first to report the inhibitory effect of glucose (in the growth medium) on the formation of bacterial deaminases (206). Later, other workers, working on bacterial deaminases, also obtained similar evidence in certain cases (262). An experiment was, therefore, set up to see if similar results were obtained in the case of A.acetigenum.

As glucose is needed for supporting the growth of the organism, it could not be completely omitted from the growth medium. Increasing amounts of glucose, however, were taken in different flasks, along with 0.5% yeast extract, for growing the culture and the turbidity of the samples drawn from each flask was measured, to get an idea of the relative growth obtained in each case after 24 hours, on a Hilger Photoelectric Absorptiometer, using the uninoculated medium as a control. The initial pH of the growth medium, before inoculation and after the growth had occurred, was also measured.

Cells were harvested from each of the flasks containing varying amounts of glucose and their relative level of deaminase activity was determined by estimating the amount of ammonia released from each of the five amino acids, tested in the usual manner, as described earlier. Aeration was provided by shaking the flasks.

It was observed that upto a certain limit, increasing amounts of glucose stimulated to a marked degree the growth of the organism. Further, the relative level of deaminase activity, in each case, was also found to be related to the amount of glucose present in the medium from which the cells were obtained and also to the physiological state of the cells of the organism as expressed by the pH of the culture at the time of harvesting the cells. This was in accordance with the observations of several workers (255). The results obtained have been given in tables no. IX to XV . From the curves drawn between deaminase activity of the cells for each of the five amino acids and the amount of glucose present in the medium at the time of growth, it would appear that increasing amountsof glucose, at the time of growth, which was also accompanied by a corresponding fall in the final pH of the growth medium, resulted in the inhibition of the deaminase activity to varying degrees in the case of all the five deaminases. The effect was more pronounced between 1% glucose and 2% glucose concentration except in the case of serine deaminase which showed greater inhibition between 2 - 4% concentration of glucose. It was further noted that the cells showed very little serine (14%) and leucine (12%) deaminase activity when the organism was grown in the presence of 4% glucose but in the case of phenyl alanine, alanine, and nor-leucine, significant deaminase activity (30%,47% and 50% respectively) was still shown by the cells even at this concentration of glucose.

TABLE NO. IX.

Effect of glucose (in the growth medium) on the relative levels of alanine deaminase activity of *A.anaticanum* 5346.

Relative deaminase activity of cells harvested from the medium containing varying amounts of glucose.

Glucose concentration in the medium	NH_3 released in* μ gms	Relative activity	Percentage inhibition
0.2%	422	100	0.0
0.6%	390	92.4	7.6
1.0%	366	86.7	13.3
2.0%	276	65.4	34.6
4.0%	200	47.4	52.6

1. Substrate- DL-αalanine.
2. * Ammonia released per 12 mg. bacterial cells (dry weight) per 3 hours at optimum temperature and pH (40°C and 7.0).
3. The activity at 0.2% glucose concentration was taken as 100 and the rest calculated on that basis.

TABLE NO. X.

Effect of glucose (in the growth medium) on the relative levels of phenylalanine deaminase activity of A.aerogenes 5346.

Relative deaminase activity of cells harvested from the medium containing varying amounts of glucose.

Glucose concentration in the medium	NH_3* released in μ gms	Relative activity	Percentage inhibition
0.2%	230	100	0
0.5%	157	71.4	28.6
1.0%	137	62.3	37.7
2.0%	96	39.1	60.9
4.0%	78	30.9	69.1

1. Substrate- DL-α phenylalanine.
2. * Ammonia released per 12 mg. bacterial cells (dry weight) per 3 hours at optimum temperature and pH (40°C and 7.4).
3. The activity at 0.2% glucose concentration was taken as 100 and the rest calculated on that basis.

TABLE NO. XI.

Effect of glucose (in the growth medium) on the relative levels of leucine deaminase activity of A.aceticanum 5346.

Relative deaminase activity of cells harvested from the medium containing varying amounts of glucose.

Glucose concentration in the medium	NH_3* released in μ gms	Relative activity	Percentage inhibition
0.2%	182.8	100	0
0.5%	137.0	74.9	25.1
1.0%	96.0	52.5	47.5
2.0%	48.0	26.2	73.8
4.0%	22.0	12.0	88.0

1. Substrate- DL- leucine.
2. * Ammonia released per 12 mg. bacterial cells (dry weight) per 3 hours at optimum temperature and pH (40°C and 7.4).
3. The activity at 0.2% glucose concentration was taken as 100 and the rest calculated on that basis.

TABLE NO. XII.

Effect of glucose (in the growth medium) on the relative levels of nor leucine deaminase activity of A.anatigenum 5346.

Relative deaminase activity of cells harvested from the medium containing varying amounts of glucose.

Glucose concentration in the medium	NH_3^* released in μ gms	Relative activity	Percentage inhibition
0.2%	238	100	0
0.5%	182	75.6	24.4
1.0%	159	66.8	33.2
2.0%	142	59.7	40.3
4.0%	120	50.4	49.6

1. Substrate- DL- nor leucine.
2. * Ammonia released per 12 mg. bacterial cells (dry weight) per 3 hours at optimum temperature and pH (40°C and 7.8).
3. The activity at 0.2% glucose concentration was taken as 100 and the rest calculated on that basis.

TABLE NO. XXII.

Effect of glucose (in the growth medium) on the relative levels of serine deaminase activity of A.aceti genum 5346.

Relative deaminase activity of cells harvested from the medium containing varying amounts of glucose.

Glucose concentration in the medium	NH_3* released in μ gms	Relative activity	Percentage inhibition
0.2%	140	100	0
0.5%	130	92.8	7.2
1.0%	116	82.8	17.2
2.0%	88	62.8	37.2
4.0%	20	14.0	86.0

1. Substrate- DL- serine.
2. * Ammonia released per mg. bacterial cells (dry weight) per 3 hours at optimum temperature and pH (45°C and 8.0).
3. The activity at 0.2% glucose concentration was taken as 100 and the rest calculated on that basis.

TABLE NO. XIV.

Effect of glucose concentrat on (in the growth medium) on the growth of A.aceticerum.

Amount of Glucose in the Medium	Initial pH of the growth medium	Final pH of the medium (after growth)	Incubation period	Optical density	Relative* growth of the organism
0.2%	6.6	5.9	24 hours	0.13	53.06
0.5%	6.6	3.8	"	0.15	61.22
1.0%	6.6	3.5	"	0.18	73.47
2.0%	6.6	3.2	"	0.22	87.75
4.0%	6.6	3.0	"	0.25	100.00

* Relative Growth of the organism at various glucose concentrations with growth at 4% glucose concentration taken as 100.

TABLE NO. XV.

Glucose concentration (in the growth medium), pH of the culture at the time of harvesting the cells and the degree of inhibition of deaminases.

Glucose concentration in the growth medium (pH = 6.6)	pH of the culture (at the time of harvesting the cells).	Degree of inhibition in the deamination of *				
		DL-α-alanine	DL-β-phenyl-alanine	DL-leucine	DL-nor leucine	L-serine
0.2%	5.9	0.0	0.0	0.0	0.0	0.0
0.5%	3.8	7.6	28.6	25.1	24.4	7.2
1.0%	3.5	13.3	37.7	47.5	33.2	17.2
2.0%	3.2	34.6	60.9	73.8	40.3	37.2
4.0%	3.0	52.6	69.1	88.0	49.6	36.2

* The deaminase activity at 0.2% glucose concentration was taken as 100, and the rest calculated on that basis.

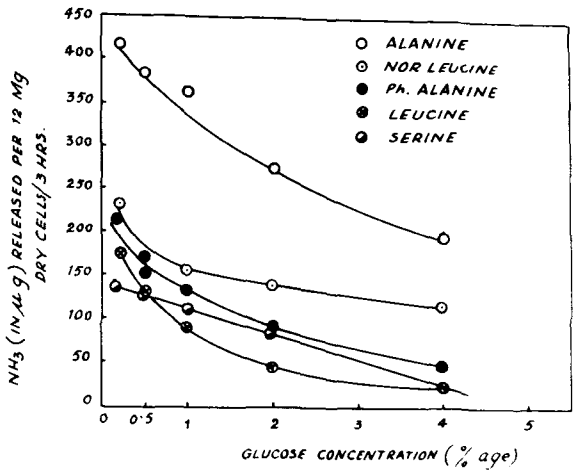


FIG. 5. EFFECT OF GLUCOSE (IN GROWTH MEDIUM) ON DEAMINATION OF AMINO ACIDS BY *A. acetigenum*. (REFER TABLE IX - XIII)

(a) Effect of age of culture on the deaminase activity of *A.sanfelicum* (NRRL).

Cells of the same culture grown for different intervals of time, when tested under the same conditions for their deaminase activity, have been shown to vary, by several workers. It was, therefore, considered worthwhile to set up an experiment to study this effect so as to know the time for which the organism should be grown to obtain a batch of cells possessing maximum activity.

Four flasks were charged with 200 ml. of the medium and incubated for 24, 48, 72 and 96 hours after inoculation. The temperature of incubation and the initial pH of the medium (6.6) was the same in all the cases. The pH of the medium was noted in each flask after the growth had occurred and it was observed that during the first twenty four hours, there occurred a fall in pH from 6.6 to 3.6 but afterwards during the remaining 72 hours, further fall in pH was very insignificant.

The relative deaminase activity of the cells obtained from each of the flasks was measured and the results obtained are tabulated in Table No. XVI. The results indicated that the best crop of cells of *A.sanfelicum* possessing maximum activity for the deamination of each of the five amino acids was obtained when the organism had been grown for

24 hours. In the case of alanine, phenylalanine and norleucine, the cells obtained after 24 hours and after 48 hours growth possessed almost the same deaminase activity. However, after 72 hours and 96 hours of growth the cells possessed a considerably reduced deaminase activity for each of the five amino acids tested.

The fall in the deaminase activity could not be possibly due to a change in pH since the activity continued to fall with the age of the culture even though there was no subsequent appreciable fall in the pH after 24 hours.

TABLE NO. XVI.

Effect of Age of Culture on the deaminase activity of A.aceticgenum.

Cells harvested from the medium (pH=6.6) after	Final pH of the Medium	µl gms. of ammonia released from [1]					Relative activity of the deaminases of [2]				
		DL-α-alanine	DL-β-Phenylalanine	DL-leucine	DL-Norleucine	DL-Serine	DL-α-alanine	DL-β-Phenylalanine	DL-leucine	DL-Norleucine	DL-Serine
24 hours	3.5	400	134	108.0	204	108	100	100	100	100	100
48 hours	3.4	392	131	84.0	200	98	98	97	77.8	98	90.7
72 hours	3.3	350	88	58.5	80.0	14	89	65.7	54.2	39.2	13.0
96 hours	3.2	280	49	35.0	48	10	70	36.6	32.3	23.5	9.3

1. NH_3 released is expressed in µl gms /12 mg. (dry weight of cells)/3 hours (with shaking the reaction mixture)

2. The deaminase activity of cells harvested after 24 hours was taken as 100, and the rest calculated on that basis.

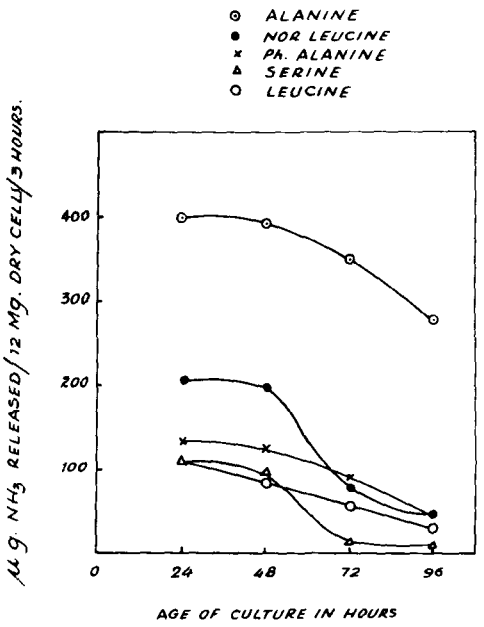


FIG. 7. EFFECT OF AGE OF CULTURE ON THE DEAMINATION OF AMINO ACIDS BY *A. acetigenum*. (REFER TABLE XVI)

(f) Effect of time of incubation on deamination by A.aceticanum

An experiment was set up with a view to finding out the time interval during which a straight line relationship existed between the deaminase activity of the resting cell suspensions of A.aceticanum and time (for which the cells were incubated with the respective amino acids and the buffer).

Six 150 ml. flasks were taken, out of which, each of the five flasks contained 5 ml. cell suspension (equivalent to 120 mg. dry weight of the cells), 10 ml. phosphate buffer of appropriate pH value and 5 ml. of the respective amino acid solution (M/20). These flasks were then mounted on a shaker and placed in a thermostatic water bath the temperature of which was set as desired. The sixth flask, which was used as a control, contained 5 ml. of water, 10 ml. phosphate buffer and 5 ml. of the cell suspension.

2 ml. of the reaction mixture was withdrawn from each of the six flasks at intervals of 0, ½, 1, 2, 3, 4, 5, 6, 7, 8 and 9 hours and its ammonia content estimated in the usual manner as described earlier. The results obtained are given in Table No.XVII after making correction for the endogenous production of ammonia (as estimated in 2 ml. of the solution from the sixth flask).

Under the conditions of the experiment, it was observed that three hours (for alanine), 5 hours (for leucine), six hours (for nor leucine) and 7 hours (for serine and phenyl alanine)

represented the time interval upto which the deaminase activity of the cells showed linear proportionality, with time. Incubation, beyond these time intervals, showed a marked decrease in deaminase activity in each case, as would appear from the plotted graph.

TABLE NO. XVII.

Ammonia production as function of time in the deamination of Amino acids by A.scaticaenum.

Incubation time in hours	µl gms* of ammonia released from				
	DL-α-alanine	DL-β-phenyl alanine	DL-leucine	DL-nor leucine	DL-serine
0.0	0.0	0.0	0.0	0.0	0.0
0.5	50	18	15	25	18
1.0	130	35	32	50	39
2.0	265	64	55	95	70
3.0	380	95	90	145	100
4.0	405	124	115	190	132
5.0	415	158	140	233	165
6.0	418	186	155	280	195
7.0	-	210	160	295	210
8.0	-	235	162	300	220
9.0	-	245	-	-	222

*
The figures represent the amount of ammonia (in µl gms.) in 2 ml. of the mixture withdrawn from each flask containing:

a) Cell suspension - 5 ml
(equivalent to 120 mg. dry weight of the cells)

b) Respective amino acid solution (M/20) - 5 ml

c) Buffer of optimum pH with respect to the amino acid being deaminated - 10 ml

The experiments were carried out at optimum temperature (for each deaminase) and under aerobic conditions, at optimum pH.

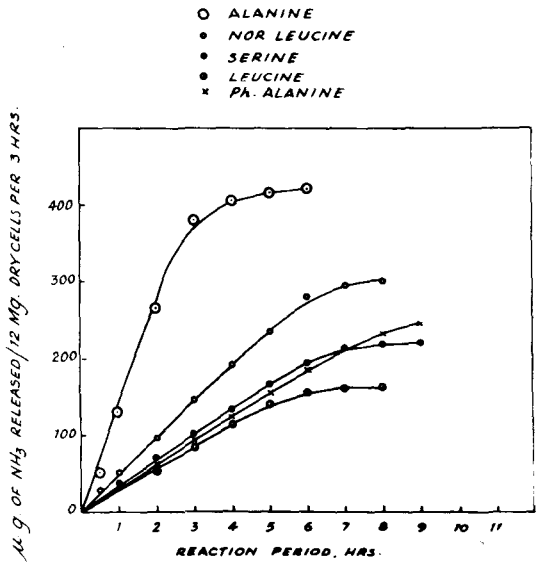


FIG.9. EFFECT OF TIME OF INCUBATION ON DEAMINATION OF AMINO ACIDS BY A. aceligenum (REFER TABLE XVII)

(g) Effect of storage of the cell suspension on the deaminase activity of A.aceticanum.

It was reported by Gale and Stephenson (206) that deaminase activity showed a decrease when the cells possessing such activity were preserved for a long time. Working on Proteus vulgaris, Stumph and Green (135), in a study of several enzymes involved in the oxidation of twenty two amino acids, also observed that all but one of these enzymes were unstable and their activities were progressively reduced as the bacterial suspension ages.

It appeared, therefore, of interest to undertake a similar study with the cell suspensions of A.aceticanum so as to obtain some information with respect to the time upto which its cells retained their deaminase activity.

Cells of A.aceticanum were obtained in the usual manner and kept in the frozen state in the refrigerator and their activity with regard to their ability to deaminate, each of the five amino acids, was tested, the same day, and after 15, 30, 45, 60 and 90 days. The results obtained are given in Table No. XVIII.

The results indicated that the deaminase activity of the cells of A.aceticanum was progressively reduced, with time, in each case. However, in the case of DL-α-alanine, the cells retained some deaminase activity even after being kept for a period of 3 months and the enzyme involved, therefore, appears to be more stable than in the other remaining four cases.

TABLE NO.XVIII.

Effect of storage of the cell suspension on the deaminase activity of A.aceticenum.

Days for which the cell suspension was kept in the frozen state	μ gms of ammonia[1] released from									
	DLαAlanine	RDA[2]	DLβPhenyl-alanine	RDA	DL-leucine	RDA	DL-Norleucine	RDA	DL-Serine	RDA
(Freshly harvested cells)	300	100	140	100	110	100	210	100	100	100
15 days	336	85.8	100	71.4	75	68.1	160	76.1	75	75
30 days	277	71.0	65	46.4	40	36.3	101	48	56	56
45 days	222	56.4	48	34.3	12	10.9	70	33	40	40
60 days	179	45.8	20	14.3	-	-	30	10	10	10
90 days	78	20.0	-	-	-	-	-	-	-	-

1. NH_3 released is expressed as μ gms/12 mg (dry weight of cells) /3 hours, at optimum temperature and pH in each case, (with shaking the reaction mixture).

2. RDA (Relative deaminase activity) = The deaminase activity of cells, harvested and tested the same day, was taken as 100, and the rest calculated on that basis.

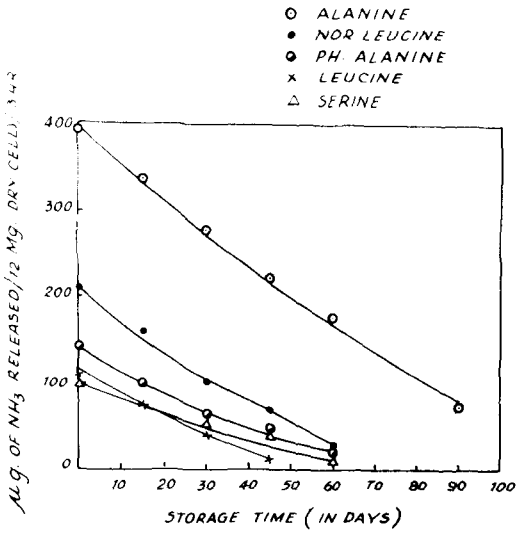


FIG. 10. EFFECT OF STORAGE OF THE CELL SUSPENSION ON THE DEAMINASE ACTIVITY OF A. acetigenum (REFER TABLE NO. XVIII)

EXPERIMENT NO. 3.

Percentage deamination of amino acids by A.acetigenum.

In an earlier experiment, 2 (f) , the effect of time of incubation, on deamination by A.acetigenum was studied and the time interval during which the deaminase activity of its resting cell suspension, with respect to each of the five amino acids, showed a linear proportionality with time was recorded. To find out the percentage of each of the amino acid deaminated during this time interval was the object of this experiment.

5 ml. cell suspension (equivalent to 120 mg. dry weight of the bacterial cells) was incubated, at optimum temperature, with 10 ml. phosphate buffer, of appropriate pH value, and 5 ml. (N/20) solution of the amino acid to be tested, for 3 - 7 hours, as the case may be, and the flasks containing the mixture mounted on a shaker. The mixture after incubation was centrifuged and 1 ml. of the centrifugate was withdrawn and its ammonia content estimated in the usual manner. The total amount of ammonia released, in each case, was then calculated by multiplying the value obtained (in 1 ml.) with 20. The total amount of the amino and ammonia nitrogen was then estimated in 15 ml., out of the remaining centrifugate, by Henriques - Sørenson Formol titration method (For details please see, Practical Physiological Chemistry by Hawk and others, 13th ed., page 897-899), and its equivalent value in 20 ml. (total incubation mixture) calculated.

On the basis of the results obtained, the total amount of each of the five amino acids initially taken and the amount of each amino acid left after deamination, it was possible to calculate the percentage of each of the amino acid deaminated. It was further confirmed by calculating from the amount of each amino acid taken, and the amount of ammonia released in each case. The results obtained are given in Table No. XIX.

TABLE NO. XIX.

Percentage deamination of Amino Acids by A.aceticerum.

Amino Acids	Amount of amino acid taken in mgms.	mgms. of ammonia released [1]	Time of incubation in hours	Amount of amino acid left after deamination in mgms. [2]	Amount of amino acid deaminated in mgms.	Percentage deamination	Percentage deamination (calculated on the basis of ammonia released)
DL.αalanine	22.25	4.00	3	1.44	20.81	93.4	94.11
DL.βPhenyl alanine	41.29	2.04	7	23.51	17.78	43.00	43.05
DL. leucine	32.79	1.3	5	22.30	10.49	31.9	30.57
DL. Nor leucine	32.79	2.3	6	15.61	17.18	52.39	51.16
DL. Serine	26.26	1.32	7	15.24	11.02	41.9	40.30

1. Total amount of ammonia released by 5 ml. cell suspension (equivalent to 120 mg. dry weight of the bacterial cells) from 5 ml. (M/20) amino acid solution, at optimum temperature and pH in each case (with shaking).

2. Calculated by Henriques - Sørenson Formol titration method.

Experiment No.4.

Non Stereospecificity of deaminases of A.aceticum.

Deaminases, like other enzymes, show specificity towards their substrates. In the oxidation of amino acids, the natural isomerides are generally attacked and the enzymes concerned are, therefore, stereospecific towards their substrates. A.suboxydans, however, was reported to be able to deaminate both the antipodes of a number of amino acids (111). Several other workers have also reported similar findings in other species of bacteria (131, 165).

An experiment was set up to study the nature of the deaminases, in A.aceticum, with respect to their ability to deaminate the DL - and the L - forms of the amino acids concerned, and to see whether they were stereospecific.

0.5 ml. solutions of L - and DL - amino acids (M/20) were added separately to the tubes containing 1 ml. phosphate buffer of appropriate pH value and 0.5 ml. cell suspension (equivalent to 12 mg dry weight of the bacterial cells). They were incubated at optimum temperature in each case, with shaking, for 3 hours. The ammonia released in each test tube was then quantitatively estimated in the usual manner and the results obtained are given in Table No. XX.

The same amount of ammonia was obtained from the same amount of L - and the DL - forms of each of the five amino acids

deaminated. The results obtained, therefore, demonstrate that the deaminases involved did not show stereospecificity towards their respective substrates. Had it not been so, the quantity of ammonia released from the DL - form would have been almost half of the amount of ammonia liberated from the L - form.

TABLE NO. XX.

Amino acid	µgms. of Ammonia released *
L-∝ alanine	356
DL-∝ alanine	308
L-β phenylalanine	121.6
DL-β phenylalanine	123.4
L- leucine	106.8
DL- leucine	108.0
L- norleucine	148
DL- norleucine	155
L- serine	92.0
DL- serine	98.0

* Ammonia released from 0.5 ml. (M/20) amino acid solution by 0.5 ml. cell suspension (equivalent to 12 mg dry weight of the bacterial cells) at optimum temperature and pH in 3 hours (with shaking).

EXPERIMENT NO. 5.

Isolation and Identification of the deamination products of amino acids deaminated by A.acetigenum.

The breakdown of amino acids, involving deamination, leads to the formation of ammonia and the corresponding fatty acids depending upon the type of deamination as already discussed, in detail, in Chapter II.

The Acetobacter species are aerobic in nature and in the experiments performed earlier, it was observed that the rate of deamination of the amino acids tested increased considerably, with the exception of serine, in an adequate supply of oxygen. It was therefore, expected that keto acids will be formed corresponding to each of the five amino acids, as a result of deamination by A.acetigenum. Serine, however, if deaminated by an oxidase system may produce hydroxypyruvic acid(219) but by a non-oxidative mechanism it also produces pyruvic acid as a result of deamination. The keto acids corresponding to each of the five amino acids tested are as follows:

Amino acid	Corresponding keto acid
Alanine	Pyruvic acid
Phenylalanine	Phenylpyruvic acid
Leucine	α-Keto isocaproic acid
Nor leucine	α-Keto nor caproic acid
Serine	Hydroxy pyruvic acid or pyruvic acid

Stumph and Green (135) reported the formation of keto acids as a result of amino acid deamination by Proteus vulgaris, isolating and identifying them as their 2 : 4 dinitro phenylhydrazones. In the present study, the same technique as was employed by these workers was adopted with slight modification.

The procedure followed is described as below:

35 ml. of the cell suspension (equivalent to 600 mgs. dry weight of the bacterial cells) were incubated with 50 ml. phosphate buffer of appropriate pH value and 25 ml. M/20 solution of the amino acid to be tested at 40°C, with shaking, except in the case of serine, the temperature of incubation for which was 45°C. At the end of the reaction (3 - 7 hours as the case may be), the mixture was centrifuged and the clear centrifugate was mixed with about 50 cc of 6 N Hcl, and recentrifuged to get a clear fluid. To this was added a filtered solution of 0.6 - 0.8% 2 : 4 dinitro phenyl hydrazine in 2NHcl which produced an immediate lemon yellow precepitate except in the case of alanine and serine.

The hydrazone obtained in each case was then purified. Since the hydrazones may, during the course of isolation, get decarboxylated giving rise to non acidic hydrazones, the solution containing the precipitated hydrazone was centrifuged and the residue was treated with a 10% solution of sodium bicarbonate so as to dissolve the acidic hydrazone leaving the non acidic hydrazone, as an impurity, which could be removed by filtration.

The scheme, as detailed in the flow sheet diagram, was followed for the isolation of the 2 : 4 dinitro phenyl hydrazones of the respective keto acids:

The reactions involved in the isolation procedure were as follows:

1. $R.CH.NH_2\ COOH \xrightarrow{O} R.CO.COOH + NH_3$

   (Amino acid) (keto acid)

2. $R.CO.COOH + H_2N.NHC_6H_3.(NO_2)_2 \xrightarrow{-H_2O} R.C.COOH$ (with $N.NH.C_6H_3(NO_2)_2$)

   (Keto acid) (2:4 dinitrophenyl-hydrazine) (2:4 dinitro phenyl hydrazone)

3. $R.C.COOH$ (with $N.NHC_6H_3(NO_2)_2$) $\xrightarrow{\text{Decarboxylation}} R.CH = N\text{-}NH.C_6H_3(NO_2)_2$

   (non acidic hydrazone)

4. $R.C.COOH$ (with $N.NH.C_6H_3(NO_2)_2$) $\xrightarrow{NaHCO_3} R.C.COONa$ (with $N.NHC_6H_3\ (NO_2)_2$)

   (acidic hydrazone) (sodium salt)

5. $R.C.COONa$ (with $N.NHC_6H_3\ (NO_2)_2$) $\xrightarrow{HCl} R.C.COOH$ (with $N.NHC_6H_3\ (NO_2)_2$) $+$ Nacl

   (Sodium salt) (2:4 dinitrophenyl-hydrazone)

The results obtained are given in Table No. XXI.

Flow sheet diagram showing the scheme employed for the isolation of 2:4 dinitrophenyl hydrazones of the keto acids obtained as a result of deamination by A.aceticerum.

The precipitate obtained after the addition of 2:4 dinitrophenyl hydrazine was washed with water and then centrifuged

Centrifugate (1), extracted with ether

Precipitate washed with 15 ml. water & centrifuged again.

Aqueous layer (rejected)

Ethereal layer, washed with water

Precipitate (2)

Centrifugate mixed with centrifugate (1)

Aqueous layer (rejected)

(Ethereal layer mixed with precipitate (2)

Treated with 10% sodium bicarbonate (20 ml.), & shaken in a seperating funnel for 35 minutes.

Ethereal layer again shaken with 5 ml. portion of sodium bicarbonate solution

Aqueous alkaline layer (3)

Ethereal layer (discarded)

Aqueous alkaline layer mixed with aqueous alkaline layer (3). Heated on a water bath, added charcoal and filtered.

The filtrate was cooled and acidified with 33% conc. Hcl, cooled and centrifuged.

Precipitate washed several times with water to remove acid, & mixed with ethereal layer (4)

Centrifugate treated with ether and washed with water.

Ethereal layer (4).

Aqueous layer (rejected)

Anhydrous Sodium sulphate was added to the above and left for 3 days, filtered & filtrate dried under reduced pressure. The residue obtained was recrystallised from benzene several times till a product (2:4 dinitrophenyl hydrazone) having constrant melting point was obtained.

TABLE NO. XXI.

Isolation of the Keto Acids as their 2:4 dinitrophenyl hydrazones as a result of amino acid deamination by A.aceticerum.

Amino Acid	Corresponding keto acid	2:4 dinitrophenyl hydrazone of the keto acid								
		Isolated= + Not isolated= -	Melting point		Analysis of carbon, hydrogen & nitrogen.					
			Correct	Found	Calculated			Found		
					C	H	N	C	H	N
					%age	%age	%age	%age	%age	%age
1. DL-αalanine	Pyruvic acid	-	-	-	-	-	-	-	-	-
2. DL-serine	Pyruvic acid or Hydroxy-Pyruvic acid	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-
3. DL-β phenyl alanine	Phenyl pyruvic acid	+	187°C	186-187°C	52.31	3.6	16.26	53.8	3.65	16.29
4. DL-leucine	α —Keto isocaproic acid	+	155-156°C	153-155°C	46.46	4.55	18.10	46.77	4.35	17.98
5. DL-nor leucine	α- Keto nor caproic acid	+	134°C	134°C	46.46	4.55	18.10	46.71	4.63	18.00

N.B. The carbon, hydrogen and nitrogen estimations were carried out by the micro-analyst of the Department of Chemistry.

EXPERIMENT NO. 6.

Attempts to isolate 2:4 dinitrophenyl hydrazones of keto acids produced as a result of deamination of alanine and serine by A.aceti.genum.

The keto acids corresponding to alanine and serine could not be isolated in experiment No. 5 and it appeared probable that the pyruvic acid, if formed, might have been further metabolised by the cells of the organism.

0.5 ml. cell suspension was incubated with 1 ml. phosphate buffer, 0.5 ml. distilled water and 1 ml. pyruvic acid (3.5 mgs.) at 40°C. A control was also set up in which no cell suspension was taken and the total volume of the reaction mixture was made upto 3 ml. by substituting 0.5 ml. more water for the cell suspension. After incubation for 3 hours, the contents of each tube were centrifuged and 2:4 dinitrophenyl hydrazine in 2N Hcl was added to the centrifugate obtained from each of the two tubes. An immediate lemon yellow precipitate was obtained in the control but no precipitate was formed in the first tube showing thereby that the cells of the organism had the ability to metabolize pyruvic acid.

It has been reported (204) that if the cell suspension of bacteria is treated with toluene and toluene is removed after 5 minutes shaking, the ability of the cells to metabolize pyruvic acid is decreased, rendering it possible to isolate the pyruvic acid.

Wood and Gunsalus (205), were able to isolate pyruvic and alpha - keto butyric acid by using vacuum dried cells of E.Coli. Both these methods were employed but attempts to isolate pyruvic acid either from alanine or from serine did not meet with any success.

The use of sodium arsenite in trapping pyruvic acid and of sodium bisulphite in trapping acetaldehyde and pyruvic acid in cultures of A.niger grown on glucose has been reported (319, 322). Experiments in which these two inhibitors were incorporated in the reaction mixture containing the cell suspension, serine or alanine, and the phosphate buffer, were performed but it was not possible to isolate pyruvic acid.

On the basis of the observations enumerated above, it seems reasonable to conclude that pyruvic acid is produced in the deamination of these two amino acids but it gets oxidised as with the result that it does not accumulate in the reacting system and cannot, therefore, be isolated as its 2:4 dinitrophenyl hydrazone.

EXPERIMENT NO. 7.

Effect of Inhibitors, Activators and Coenzymes on amino acid deaminases of A.acetigenum.

A.acetigenum, as seen in earlier experiments, was found to be able to deaminate five amino acids. It was further observed that the rate of deamination of alanine, phenyal alanine and nor leucine was increased in an adequate supply of oxygen. However, this effect was not noted in the deamination of serine.

In subsequent work, therefore, the effect of inhibitors and activators was only seen with respect to the enzyme systems, involved in the deamination of DL-∝alanine and DL-serine, representing the two main types.

A reference to the literature on the subject shows that deamination of a large number of amino acids including alanine, phenyl alanine, leucine and nor-leucine is brought about by a non-specific L- or D- amino acid oxidase depending on the type of isomer used as the substrate. Aspartic acid, glycine and glutamic acid, however, are deaminated by specific enzymes corresponding to these amino acids. Serine and other hydroxy amino acids and SH-containing amino acids are deaminated by other type of enzymes called dehydrases or desulphydrases respectively. The coenzymes or prosthetic

group of amino acid oxidases have generally been shown to be FAD or FMN but very little information is available concerning the coenzymes of amino acid oxidase in bacteria. The difficulty in this regard is mainly due to the firm attachment of the coenzymes (FAD or FMN) to the protein so that the usual resolution methods for separating the two are not successful making it difficult to observe the activation of the enzyme on the addition of coenzymes. However, it has been reported, in several cases, that appreciable activation of deaminases was observed when the coenzymes were added even to the cell suspension.

The effect of some organic and inorganic compounds, some of which react with active carbonyl groups, are metal binding agents or contain an active sulfhydryl group in the molecule, was investigated on the enzyme activity under identical conditions. Effect of other inhibitors, that have already been reported to be effective in the inhibition of amino acid deaminases, was also studied so as to obtain some evidence with respect to the nature of the active groups present on the surface of the enzyme/enzymes.

(i) Effect of various organic and inorganic inhibitors on the deamination of DL alanine.

The effect of various inhibitors was investigated under identical conditions. All the compounds, before being used, were tested for their purity. The solutions, prepared in

distilled water, were of the concentrations as given in the table. Octyl alcohol, caprylic alcohol and toluene were distilled and then added, as such, in volumes of 0.5 ml.

The cell suspension (0.5 ml.), equivalent to 12 mg. dry weight of bacterial cells was preincubated, for five minutes at 40°C, with 0.1 M phosphate buffer (1 ml.) of pH 7 and 0.5 ml. of a neutral solution of the respective compounds in increasing concentrations as detailed in table No.XXII, with adequate shaking. DL α alanine solution (0.5 ml.), equivalent to the amount as given in the table, was then added and the final volume, in each tube, was made to 3 ml. by the addition of distilled water. In every set of experiment, a standard was also run, in which the solution of the compound, under test, was replaced by the same volume of water i.e. 0.5 ml. The incubation was done at 40°C, with shaking, for three hours, after which the reaction was stopped by the addition of 30% trichloroacetic acid (0.5 ml.), and the ammonia released in each case was estimated by the method, already described. The results, as given in the table, represent micrograms of ammonia released per 12 mg. dry weight of bacterial cells per 3 hrs. and were obtained by correcting for the endogenous ammonia produced. % age inhibition of deaminase activity, was then calculated by taking the activity in the standard (without the compound whose effect was being tested) as 100, and calculating the rest on that basis.

TABLE NO. XXII.

Effect of various Organic and Inorganic inhibitors on the deamination of D(-) α alanine by A.aceticanum.

Name of the Compound	Amount added in μ moles (in 0.5 ml.)	μ gms. of ammonia released	Relative deaminase activity	% age inhibition
	nil	400	100	-
	0.5	220	55	45
NaCN	1.0	170	45	55
	5.0	94	23.5	76.5
	50.0	72	18.0	82.0
	nil	400	100	-
	0.5	325.5	88.5	11.5
Hydrazine Sulphate	1.0	209.65	52.4	47.59
	5.0	156.32	39.08	60.92
	50.0	18.39	4.59	95.41
	nil	400	100	-
	0.5	282	70.5	29.5
Hydroxylamine	1.0	217	54.25	45.75
	5.0	204	51.0	49.0
	50.0	180	45.0	55.0

Continued.....

Name of the Compound	Amount added in µ moles (in 0.5 ml.)	µ gms. of ammonia released	Relative deaminase activity	% age inhibition
Semicarbazide hydrochloride	nil	400	100	-
	0.5	373.5	93.37	6.63
	1.0	369.0	92.25	7.75
	5.0	366.0	91.50	8.50
	50.0	350.7	87.60	12.40
Sodium bisulphite	nil	400	100	-
	0.5	301.5	75.38	24.62
	1.0	278.4	69.60	30.40
	5.0	262.7	65.67	34.33
	50.0	0	0	100.00
$CUSO_4$, 5 H_2O	nil	400	100	-
	0.5	224.0	55.57	44.43
	1.0	175.88	43.64	56.63
	5.0	123.43	30.62	69.38
	50.0	0	0	100.00
$MgSO_4$, 7 H_2O	nil	403	100	-
	0.5	390.9	96.9	3.1
	1.0	381.5	94.6	5.4
	5.0	381.5	94.6	5.4
	50.0	383.45	95.1	4.9

Continued.....

Name of the Compound	Amount added in μ moles (in 0.5 ml.)	μ gms. of ammonia released	Relative deaminase activity	% age inhibition
$ZnSO_4$, 7 H_2O	nil	403	100	-
	0.5	281.16	69.76	30.24
	1.0	268.44	66.61	33.39
	5.0	246.48	61.16	38.84
	50.0	246.50	61.20	38.80
$HgCl_2$	nil	403	100	-
	0.5	23.43	5.81	94.19
	1.0	14.99	3.71	96.29
	5.0	0	0	100.00
$AgNO_3$	nil	400	100	-
	0.5	128	32	68
	1.0	20	5	95
	5.0	0	0	100
Sodium arsenite	nil	402	100	-
	0.5	331	82.4	17.6
	1.0	290	72.13	27.87
	5.0	253	65.42	34.58
	50.0	180	45.80	55.20

Continued.....

Name of the Compound	Amount added	µ gms. of ammonia released[1]	Relative deaminase activity	% age inhibition
	nil	398	100	-
OCTYL ALCOHOL	0.5 ml.	0	0	100
CAPRYLIC ALCOHOL	0.5 ml.	0	0	100
TOLUENE	0.5 ml.	0	0	100
	nil	198	100	-
	0.5 µ moles	219	110	(10% activation)
$MnCl_2, 4H_2O$	1.0 µ moles	280	141.4	(41.4% ")
	2.0 µ moles	288	145.4	(45.4% ")

1. Ammonia released per 12 mg. bacterial cells (dry weight) per 3 hrs. at 40°C, pH 7, with shaking except in the case of manganese chloride, where the quantity of cells taken was 5 mg.

2. The amount of DL-α alanine taken, in each case, was 2.225 mgs. (0.5 ml. of M/20 solution).

3. The activity was taken as 100 in the standard (which did not have the compound whose effect was being tested) and the relative deaminase activity in each case was calculated on that basis.

The results obtained, as presented in Table No.XXII, show that of the various carbonyl group reagents tested, Sodium bisulphite was able to inactivate completely the enzyme when added in a concentration of 50 μ moles / 3 ml. of the reaction mixture. Even at lower concentrations, its inhibition of the deaminase activity was perceptible. The other reagents also exercised a marked effect on deaminase activity except in the case of Sodium Carbazide which could not produce any appreciable inhibition. Sodium cyanide, an unspecific inhibitor, inactivated the enzyme to an extent of 82% when used in a concentration of 50 μ moles / 3 ml. of the reaction mixture. Sodium arsenite D which was found to be without any effect on the deamination of amino acids by Proteus vulgaris (135) was, however, able to inhibit the deamination of alanine at the various levels of concentration employed. As reported in amino acid deaminases of various other species (118, 135), the activity of the deaminase in A.aegaticanum was completely lost on the addition of Octyl alcohol, Caprylic alcohol and Toluene.

Of the various metal ions tested, Mg^{++} was without any effect, Zn^{++} inhibited the enzyme to an extent of 38.8% (on the addition of 50 μ moles of $ZnSO_4$/3ml. of the reaction mixture) and heavy metals viz. Hg^{++}, Ag^{+} and Cu^{++} inhibited the deamination of alanine even at low concentrations. Mn^{++}, however, was able to progressively activate the enzyme at different levels of concentration, as would appear also from the Table No.XXII, and activated it to about 45% on the addition of 2 μ moles / 3 ml. of the reaction mixture.

(11) Effect of carbonyl group reagents on the deamination of DL-Serine by A.aerogenum.

Serine dehydrase (deaminase) has been shown to be inhibited by carbonyl group reagents by several workers in most of the microorganisms (131, 212, 220). In the present investigation, the effect of hydroxylamine, sodium bisulphite, and semicarbazide hydrochloride was studied on the deamination of DL-serine. The effect of sodium cyanide was also studied. The experiment was planned in exactly the same manner as described fully in the case of DL-α alanine (page 118).

The results obtained, as presented in Table No. XXIII, indicate that these inhibitors did inhibit, to some extent, the deaminase activity but none of the inhibitors tested could completely inhibit the enzyme activity even at concentrations as high as 50 μ moles / 3 ml. of the reaction mixture. In the case of sodium cyanide, however, 55.56% inhibition of deaminase activity was possible when it was added at a concentration of 50 μ moles / 3 ml. of the reaction mixture.

TABLE NO. XXIII.

Effect of some inhibitors on the deamination of DL-serine by A.aerogenum.

Name of the Compound	Amount added in µ moles (in 0.5 ml.)	µ gms. of ammonia released[1]	Relative deaminase activity	% age inhibition
	nil	108	100	-
	0.5	94	87.0	13.0
Hydroxylamine	1.0	84	77.8	22.2
	5.0	70	64.8	35.2
	50.0	55	51.0	49.0
	0.5	92	85.2	14.8
Sodium bisulphite	1.0	80	74.1	25.9
	5.0	64	59.3	40.7
	50.0	60	55.6	44.4
	0.5	95	88	12
Semicarbazide Hydrochloride	1.0	86	80	20
	5.0	67	63	37
	50.0	50	46.3	53.7
	0.5	72	66.7	33.3
Sodium cyanide	1.0	60	55.6	44.4
	5.0	53	49.1	50.9
	50.0	48	44.4	55.6

1. Ammonia released / 12 mg. bacterial cells (dry weight)/ 3 hrs. at 45°C, pH 8, with shaking.

2. Amount of DL-Serine = 2.6267 mg.(0.5 ml of M/20 solution).

(iii) Effect of Metal binding agents on the deamination of DL-∝ alanine and DL-serine by A.aceticgenum.

Serine dehydrase (204, 294, 321) and some other amino acid deaminases (176, 309) have been reported to be associated with a metal ion. It should, therefore, be possible to inactivate them in presence of a metal binding agent.

It was with this idea that the effect of 8- hydroxy quinoline, ∝∝ - dipyridyl and ethylene diamine tetra acetic acid (EDTA) was studied on the deamination of DL-∝ alanine and DL-serine by A.aceticgenum.

The effect of increasing concentrations of these metal binders on deamination was studied with a view to ascertain if they caused any inhibition in the deamination process. The details of the experiment were as follows:

Cell suspension (0.5 ml.), equivalent to 12 mg dry weight of the bacterial cells, 1 ml. buffer (M/10), of appropriate pH value (7 for alanine and 8 for serine), 0.5 ml. of the respective inhibitor, in concentrations as given in Table No. XXIV and 0.5 ml. (M/20) solution of each of the two amino acids were incubated for 3 hours at 40°C, in the case of alanine, and 45°C in the case of serine, with shaking. The ammonia released in each case was then estimated as usual. Tubes which

contained all the other components, except the inhibitor, were treated in a similar manner and served as control for each set of experiment.

In another experiment, the results of which are presented in Table No. XXV, 0.5 ml. cell suspension, equivalent to 12 mg. dry weight of the bacterial cells, was incubated with 0.5 ml. of the respective inhibitor solution (5 μ moles) at 37°C for 1,2,3 and 4 hrs. At the end of these time intervals, 1 ml. phosphate buffer of appropriate pH value was added along with 0.5 ml. of the amino acid solution (M/20) and further incubated for 3 hrs., with shaking, at 40°C(for alanine) and at 45°C (for serine). The ammonia released in each case was then assayed as usual. This experiment was set up to see if the time of contact (of the inhibitor with the cells) affected in any way the process of deamination.

The deaminase activity was taken as 100 in the standard (which did not have the inhibitor whose effect was being tested) and the relative deaminase activity, in each case, was calculated on that basis.

The results obtained clearly showed that the metal binding substances tested had an adverse action on the enzyme activity. 8- hydroxy quinoline and EDTA appeared to be more potent inhibitors then $\alpha\alpha$- dipyridyl in the case of both the amino acids. It was further observed that their inhibitory effect on the deamination of serine was more pronounced than on the deamination of alanine. The degree of inhibition was also found to depend to a large extent on the time of contact of the inhibitor with the cell suspension.

TABLE NO. XXIV

Effect of metal binding agents on the deamination of DL-α alanine and DL-serine by A.acetigenum. (different concentrations)

Name of the metal binding agent	Amount taken in µ moles	µ gms of NH_3 released from alanine	Relative deaminase activity	% age inhibition	µ gms of NH_3 released from serine	Relative deaminase activity	% age inhibition
8- HYDROXY QUINOLINE	nil	400	100	-	108	100	-
	0.1	380	95	5	90	83.3	16.7
	0.25	336	84	16	81	75	25
	0.50	302	78	22	74	68.5	31.5
	1.00	272	68	32	46	42.5	57.5
	5.00	160	40	60	20	18.5	81.5
α α-DIPYRIDYL	nil	400	100	-	112	100	-
	0.1	390	97.5	2.5	95	84.8	15.2
	0.25	355	88.7	11.3	88	78.5	21.5
	0.5	342	85.5	14.5	81	72.3	27.7
	1.0	300	75.0	25.0	62	55.3	44.7
	5.0	210	52.5	47.5	44	39.2	60.8
ETHYLENE DIAMINE TETRA ACETIC ACID (EDTA)	nil	398	100	-	108	100	-
	0.1	382	95.5	4.5	92	85.1	14.9
	0.25	340	85.4	14.6	83	76.8	23.2
	0.5	300	75.3	24.7	76	70.3	29.7
	1.0	275	69.0	31.0	50	46.3	53.7
	5.0	170	42.7	57.3	20	18.5	81.5

TABLE NO. XXV

Effect of metal binding agents on the deamination of DL-α alanine and DL-serine by A.aceticgenum.
(Time of contact)

Name of the metal binding agent	Time of contact with the cell suspension before incubation with substrate (in hrs)	μ gms of NH_3 released from alanine	Relative deaminase activity	% age inhibition	μ gms of NH_3 released from serine	Relative deaminase activity	% age inhibition
	-	400	100	-	110	100	-
	1	112	28	72	18	16.4	83.6
8- HYDROXY QUINOLINE	2	85	21.3	78.7	5	4.5	95.5
	3	15	3.8	96.2	0	0	100.0
	4	0	0	100	-	-	-
	-	400	100	-	110	100	-
	1	202	50.5	49.5	41	37.3	62.7
$\alpha\alpha$-DIPYRIDYL	2	115	28.8	71.2	29	26.4	73.6
	3	103	25.8	74.2	15	13.6	86.4
	4	107	26.8	73.2	0	0	100.0
	-	400	100	-	110	100	-
	1	150	37.5	62.5	16	14.5	85.5
ETHYLENE DIAMINE	2	99	24.8	75.2	8	7.3	92.7
TETRA ACETIC ACID	3	33	8.3	91.7	0	0	100.0
(EDTA)	4	0	0	100.0	-	-	-

(iv) Effect of Biotin on the deamination of DL-∝ alanine and DL-serine by A.aceticenum.

Lichstein and Umbreit (208, 209) presented some evidence to show that biotin, in some form, was able to function as a coenzyme in the deamination of serine and threonine by P.vulgaris, Bacterium cadaveris and E.coli. Other workers also obtained results (305, 317) which supported the involvement of biotin as a cofactor in the deamination of aspartic acid, serine and threonine by resting cells of bacteria. Deamination of aspartic acid, glutamic acid and alanine by Neurospora mycelium was also shown to be activated by the addition of biotin (318).

An experiment was, therefore, set up to ascertain if biotin played any role in the deamination process of alanine and serine by A.aceticenum.

The technique adopted was the same as reported by Lichstein and Umbreit (208). The living cells of the organism were exposed to phosphate buffer (1 molar) at pH 4, for a period of 30 minutes, at 37°C. This was done to inactivate the deaminase of the bacterial cells so as to see if the inactivation could be reversed on the addition of biotin.

The cells treated in the manner given above, so as to inactivate the deaminases, were then incubated with either of these two amino acids and buffers of appropriate pH values, with and without the addition of 100 µ gms. of biotin. The ammonia

released in both the cases was then estimated, in the manner as described earlier, and compared with the ammonia released under identical conditions when unbreated cells were used.

The results,as given in Table No. XXVI clearly demonstrated that the amount of ammonia released was reduced in the case of both the amino acids that is alanine and serine when treated cells were used. The addition of biotin, however, did not bring about any reactivation of the enzymes in either case.

On the basis of these observations, it seems reasonable to conclude that biotin is not involved in the deamination of serine and alanine by A.aceatigenum.

TABLE NO. XXVI.

Effect of Biotin on the deamination of DL-α alanine and DL-serine by A.aceticgenum.

	μ gms of ammonia released from	
	Alanine	Serine
1. Control (untreated cells without biotin)	400	107
2. Treated cells (without biotin)	301	64.5
3. Treated cells (with 100 μ gms. biotin)	299.5	65.5

1. Ammonia released is expressed as μ gms/12 mgs. dry weight of the bacterial cells/3 hrs. at 40°C and pH 7, in the case of alanine and at 45°C and pH 8, in the case of serine, with shaking.

2. The cells were treated by exposing them to phosphate buffer (1 M) at pH 4 for a period of 30 minutes at 37°C.

(v) Effect of some vitamins on the deamination of DL- ∝ alanine by A.aceticgenum.

Very little information is available with respect to the nature of the prosthetic group of bacterial amino acid oxidases. Stumph and Green (135), however, reported that this enzyme appeared to be a flavoprotein. Alanine has also been reported to be deaminated by a NAD dependant alanine dehydrogenase (314). A NADP dependant phenyl alanine dehydrogenase in E.coli has also been reported (315). Alanine deaminase of Rhodo pseudomonas spheriodes has been regarded as Pyridoxal phosphate dependant by D.S.Hoare (131). Riboflavin phosphate has been shown to be the coenzyme of mammalian L-amino acid oxidase (316).

An attempt was made to investigate the nature of the coenzyme involved in the deamination of DL- ∝ alanine brought about by A.aceticgenum. Only pyridoxine, pyridoxamine, pyridoxal phosphate and riboflavin phosphate were available and were tested for their activity in the deamination of this amino acid. Since the experiments were performed with intact cells, the experiments were repeated after about 2 months, using aged cell suspension also.

The results obtained are given in Table No. XXVII. None of the vitamins used showed any noticeable effect. As stated earlier, since cell free enzyme preparations were not used, no satisfactory conclusion could be drawn from these observations.

TABLE NO. XXVII

Effect of some vitamins on the deamination of DL-α alanine by A.aceti genum.

Vitamin added	µl gms of ammonia released from 0.5 ml. (M/20) solution of alanine by 0.5 ml. cell suspension (equivalent to 12 mgs. dry weight of the cells) in 3 hrs. at 40°C, and pH 7, with shaking.
1. None	401
2. Pyridoxal phosphate (100 µl gm.)	392
3. Pyridoxamine (100 µl gm.)	394
4. Pyridoxine (100 µl gm.)	380
5. Riboflavin phosphate (100 µl gm.)	403

N.B.: No significant activity was noticed even when 2 months old cell suspensions were used.

(vi) Effect of gassing with nitrogen gas, addition of cysteine, riboflavin phosphate, pyridoxine pyridoxamine and pyridoxal phosphate on deamination of Dl- and L-serine by A.aerogenes.

Deamination of serine in most of the biological systems has been found to be stimulated by PLP (207, 220). Out of the two systems active in E.coli for the deamination of Dl-serine, one which is active for D-form requires PLP as coenzyme and the other, which is active for L-serine, requires AMP and reduced glutathione. Anaerobic reduced condition has also been reported to be stimulatory, in certain cases, in the deamination process (256).

In view of the above reports in literature, the present experiment was set up to investigate the effect of these factors on the deamination of Dl-serine by A.aerogenes.

Cells, fresh as well as old (60 days old cells which had lost most of their deaminase activity) were taken and the effect of the substances listed above was observed using L and Dl- forms of serine as substrates. The results obtained are tabulated in Table No.XXVIII. Anaerobic condition was produced by gassing the reaction flask with nitrogen gas for a period of 10 - 15 minutes. Similarly, reduced condition was obtained by addition of 100 λ gms. of

cysteine monohydrochloride to the reacting system. Riboflavin phosphate, pyridoxine, pyridoxamine and PLP were added in quantities as indicated in Table No. XXVIII.

The results obtained clearly indicate that deamination of L-serine was not affected by any of the additions made nor was it changed to any significant extent in an atmosphere of nitrogen. Similar results were obtained in the deamination of DL-serine when fresh cells of the organism were used. However, PLP was able to activate to a slight extent the deamination of DL-serine by old cells. On the basis of these observations, it seems reasonable to suppose that the deamination of DL-serine is perhaps brought about by two enzyme systems, D - serine and L - serine dehydrase. The former is PLP dependant while the latter is not. Further, the results show that PLP cannot be replaced by its analogues i.e. pyridoxine and pyridoxamine. Anaerobic and reduced condition was also not able to show any change in the pattern of the results obtained. Similarly riboflavin phosphate also did not appear to show any effect as has been reported in the case of other micro organisms.

TABLE NO. XXVIII.

Effect of gassing with nitrogen gas, addition of cystein, riboflavin phosphate, pyridoxine, pyridoxamine and pyridoxal phosphate on deamination of Dl- and L- serine by A.aerogenes.

Addition	NH_3 released in U gms/12 mg. dry cells/ 2 hours from			
	Diserine		Lserine	
	Fresh cells	60 days old cells	Fresh cells	60 days old cells
None	109	15	101	13
Gassing with nitrogen gas	107	16	100	14
Cysteine-HCl (100 U gms)	105	14	99	14
Pyridoxine (100 U gms)	108	14	98	13
Pyridoxamine (100 U gms)	107	15	99	13
Riboflavin phosphate (100 U gms)	105	14	100	14
Pyridoxalphosphate:				
100 U gms	108	40	100	12.8
180 U gms	107	43	-	-

SUMMARY OF RESULTS AND DISCUSSION

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1. Out of the sixteen species of Acetobacter tested for their ability to deaminate amino acids, only thirteen produced ammonia from the various amino acids taken as their substrate. Three species viz. A. ascendens, A. oxydans and A. pasteurianum, however, did not deaminate any of the amino acids tested. Similarly, the amino acids which were not deaminated by any of the species tested in the present investigation were, L-tyrosine, L-histidine, L-cysteine hydrochloride, L-cystine, L-lysine monohydrochloride, L-Arginine monohydrochloride, DL-isoleucine, DL-β-alanine and Dl-tryptophane.

2. A. aceti (5265 NCIB) appeared to be particularly promising and was found to deaminate Dl-serine, Dl-norleucine, Dl-leucine, Dl-alanine, Dl-β-phenylalanine, and L-asparagine.

3. Factors affecting the deamination of amino acids by A. aceti (5265) were studied and it was found that:

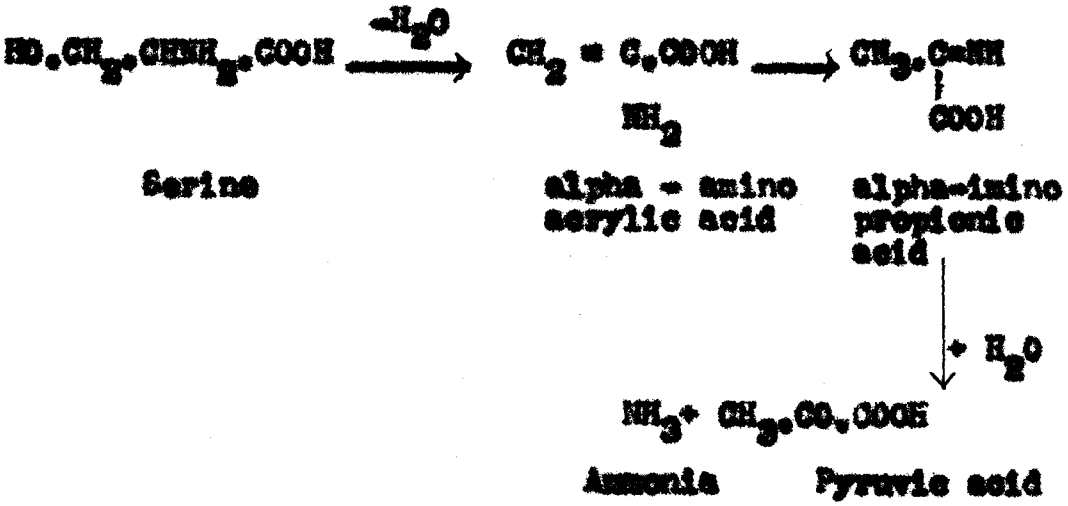
(a) The pH optima for the deamination of DL-alanine and DL-norleucine were 7 and 7.8 respectively, 7.4 in the case of DL-phenylalanine and DL-leucine, and 8.0 in the case of DL-serine (Fig. 2). This

was in accordance with the observations of earlier workers who have reported that the optimum pH range at which deaminases function is usually from 7 - 9.

(b) 40°C was found to be the optimum temperature for alanine, phenylalanine, leucine and norleucine deaminases, and 45°C for serine deaminase (Fig. 3). The optimum temperature for serine deaminase in A.aerogenes appears to be rather higher than found for most of the bacterial deaminases. Though the biological reactions are generally carried out at 37°C, deamination has also been shown to occur from 46°C to 52°C, or even higher, as reported in the case of threonine deaminase of sheep liver (295). The results obtained further indicated that the optimum temperature for deamination was higher than that at which the organism was grown i.e. 20° - 35°C. The deamination of DL-serine by Lactobacillus fermi, controlled by two enzyme systems, was shown to have its optimum above the temperature of the growth of that organism (255). One system was shown to produce maximum release of ammonia at 45°C, and the other at 55°C. The findings, therefore, in the present study were almost similar to those reported by other workers.

(e) With the exception of DL-serine, where no appreciable difference was observed, in the remaining four amino acids, the ammonia produced as a result of amino acid deamination was much more when the flasks were subjected to shaking than otherwise when they were kept stationary. A reference to Fig. 4 will clearly bring out the fact that DL-alanine was most affected, while in the case of DL-norleucine, DL-phenylalanine and DL-leucine, the effect was comparatively less, and almost negligible in the case of DL-serine. It is quite likely, therefore, that the deaminases, as they occur in *A.hantzianum*, may be of two types viz. oxidative and non-oxidative, which would explain the observations made. The deamination of DL-serine was perhaps of the non-oxidative type. It has been reported that in biological systems, serine is deaminated in two ways: oxidatively to give hydroxy-pyruvic acid (219) or pyruvic acid (286) with an uptake of oxygen; and non-oxidatively (114) to produce pyruvic acid but without any net oxygen utilisation which corresponds to a non-enzymic PLP

catalysed reaction (333) and is carried out by D-serine dehydrase as shown by Metzler and Snell (207). Since enzymic deamination of DL-serine in the present study was not activated in an adequate supply of oxygen, it might be postulated that the deamination of serine is brought about through dehydrative deamination as proposed by Chargaff and Sprinson (134):



The favourable effect of aeration on deamination of the rest of the amino acids, alanine, phenylalanine, leucine and norleucine by *A.aerogenes* was perhaps due to the usual oxidative mechanism as follows:

The isolation of the corresponding keto acids as done in Experiments No. 5 and 6, further supported this assumption.

(d) Upto a certain limit, increasing amounts of glucose stimulated to a marked degree the growth of A.aerogenes. The relative level of deaminase activity in each case was found to be related to the amount of glucose present in the medium from which the cells were obtained, and also to the physiological state of the cells of the organism, as expressed by the pH of the culture at the time of harvesting the cells. Increasing amounts of glucose at the time of growth, which was also accompanied by corresponding fall in the final pH of the growth medium, resulted in an inhibition of the deaminase activity to varying degrees in the case of all the five deaminases (Fig. 5).

It has been well known that in growing cultures of bacteria, carbohydrate exerts a sparing action on the decomposition of proteins and amino acids. The data as presented in Table No. (IX - XIII) also indicate clearly that the presence of carbohydrate

(Glucose) in increasing concentrations reduced the deaminase activity of the cells. The effect, however, varied for each amino acid as can be seen from the percentage inhibition of the deaminase activity in the case of each of the five amino acids (Table No. XV). Various reasons have been assigned to the fall of deaminase activity in presence of increasing amounts of glucose. Gale (122) was able to show that the fall in deaminase activity was neither due to anaerobiosis produced by the evolution of the fermentation gas nor could it be due to the acid formation from glucose during growth. Later, however, some Japanese workers (324) obtained evidence to support the view that fall in deaminase activity was actually due to the excess of acid production from glucose, in the growth medium. Cells of Proteus vulgaris grown in presence of glucose were shown by them to have a reduced deaminase activity for the deamination of L-phenylalanine. Similarly, since Bacterium denitrificans (127) did not produce acid from glucose, the effect on deamination, in varying concentrations of glucose, was negligible in this case. In the present study, the fall in the respective

amino acid deaminase activity of A.sanguineum does not seem to be possibly due to a change in pH since activity continued to fall with increasing amounts of glucose even though there was no significant fall in the pH after 0.6% addition of glucose (see Table No. XV).

(c) Cells of A.sanguineum, grown for different intervals of time, when tested under the same conditions, were found to vary in their deaminase activity (Fig. 7). The best crop of cells of A.sanguineum, possessing maximum activity for the deamination of each of the five amino acids, was obtained when the organism had been grown for 24 hours. The relative deaminase activity of cells obtained after 24 hours growth, and after 48 hours growth, remained almost constant with respect to alanine, phenylalanine and norleucine but showed some variation in the case of leucine and serine. After growth had taken place for 72 and 96 hours, the cells obtained exhibited a considerably reduced deaminase activity for each of the five amino acids tested.

The fall of deaminase activity with the age of culture has been reported by several workers (173, 206, 266). Srikantan, Agarwala and Shrivastava (300)

also obtained some what similar results in their studies with the enzyme make up of Pasteurella pestis when it was shown that an equal rate of oxidation was shown by 24 and 48 hours old cultures of the said organism but there was a considerable drop in the rate of oxidation after the organism had been grown for 96 and 144 hours.

The fall in the deaminase activity could not possibly be due to a change in pH since the activity continued to fall, with the age of the culture, even though there was no subsequent appreciable fall in the pH after 24 hours (Table XVI).

(f) Under the conditions of the experiment, it was observed that 3 hours for alanine, 5 hours for leucine, 6 hours for norleucine and 7 hours for serine and phenylalanine represented the time intervals upto which the deaminase activity of the cells of A.anatigenum showed linear proportionality with time (Fig. 9). Incubation beyond these time intervals showed a marked decrease in deaminase activity in each case. The percentage deamination, corresponding to these time intervals, has been tabulated

in Table No. XIX and was 94%, 43%, 30.6%, 54.2%, and 40.8% in the case of alanine, phenylalanine, leucine, nor-leucine and serine respectively.

(g) Cells of *A.saccharigenum*, when stored for various intervals of time, showed a progressive reduction in their deaminase activity (Fig. 10). However, in the case of Dl-alanine, the cells retained some deaminase activity even after being kept in the frozen state for a period of three months and the enzyme involved, therefore, appears to be more stable than in the case of the remaining four amino acids. Reduced deaminase activity on storage has been reported by Gale and Stephenson (200), Stumph and Green (135), and several other workers working with the amino acid oxidation of *B.bulgaris*. It was shown by Stumph & Green that the cells could oxidise only 11 out of the 22 amino acids, which they could attack originally, when kept at 0.°C for a period of two weeks. Further storage did not reduce the number of amino acids to be deaminated, but the rate of oxidation was certainly reduced.

4. The data presented in Table No. XX show that both the optical antipodes of alanine, phenylalanine, leucine, norleucine and serine were almost equally deaminated by cell

suspensions of A.aceticaenum. The deaminases involved, therefore, appear to be non-stereospecific towards their substrates; or perhaps D-and L forms of the amino acids were being deaminated by different enzymes present in the suspension. However, it is quite likely that the D-form is racemised first to the L-form by amino acid racemase and then gets deaminated. Due to the non-availability of D-amino acids, it was not possible, however, to draw a more definite conclusion from these observations.

The ability of an organism to deaminate both the optical isomers of an amino acid has been reported by several workers. Stokes and Alma Larsen found that both the forms of alanine, serine and aspartic acid were oxidised by Acetobacter suboxydans (111). B.pyocyaneus (Ps.aeruginosa) was shown to be able to oxidise and deaminate both the isomers of alanine, serine, tyrosine and proline (165). Similarly, B.Proteus was also found to have the ability to oxidise both the optical isomers of alanine and serine (164). Cells of Bact. coli were shown to be effective in the deamination of both, D-and L-serine (206). More recently, it has been reported that crude cell free extracts of Rhodopseudomonas spheroides deaminated both D-and L-serine. The observations made, therefore, in this respect, were not unusual in the light of the reports of earlier workers.

5. The deamination products of the amino acids deaminated by *A.aerogenes* were isolated and identified. The keto acids corresponding to leucine, norleucine and phenylalanine were isolated and identified as their 2, 4 dinitro phenyl hydrazones. (See Table No. XXI). The keto acids corresponding to alanine and serine, however, could not be isolated.

6. Indirect evidence was obtained to suggest that pyruvic acid is perhaps produced as a result of deamination of these two amino acids but it gets oxidised *par se* with the result that it does not get accumulated in the reacting system and could not, therefore, be isolated as its 2, 4 dinitrophenylhydrazone.

7. The effect of various organic and inorganic inhibitors on the deamination of Dl-alanine by *A.aerogenes* was observed.

The results obtained, as presented in Table No.XXII, indicated that inhibition of alanine deaminase occurred to a significant extent when carbonyl group reagents were taken in various concentrations. Of the various carbonyl group reagents tested, sodium bisulphite was able to inactivate completely the enzyme when added in a concentration of 30 μ moles per 3 ml.

of the reaction mixture. Even at lower concentrations, its inhibition of the deaminase activity was perceptible. The other reagents viz. sodium cyanide, hydrazine sulphate and hydroxylamine also exercised a marked effect on deaminase activity except sodium carbazide which could not produce any appreciable inhibition. The results obtained, therefore, provided an indirect evidence of the presence of carbonyl group on the surface of the enzyme which was essential for the activation of the substrate.

The activity of alanine deaminase was also completely lost on the addition of octyl alcohol, caprylic alcohol and toluene.

Of the various metal ions tested, Mg^{++} was without any effect, Zn^{++} inhibited the enzyme to an extent of 38.3%, on the addition of 50 µ moles of $ZnSO_4$ /3 ml. of the reaction mixture, and heavy metals viz Hg^{++}, Ag^{+}, and Cu^{++} inhibited the deamination of alanine even at lower concentrations. Mn^{++}, however, was able to activate progressively the enzyme at different levels of concentration, as would appear from the data presented in Table No. XXII, and activated it to about 45% on the addition of 2 µ moles/ 3 ml. of the reaction mixture.

The L-amino acid oxidase of Proteus vulgaris was also found to be inhibited by $AgNO_3$, $CuSO_4$, $HgCl_2$, caprylic alcohol and sodium cyanide by Stumph and Green (135). Arsenite which is usually not found to inhibit most of the amino acid oxidases, as also in the case of Proteus vulgaris, inhibited the deamination of alanine by A.aceticgenum. However, the oxidation of alanine and other amino acids by Pasteurella pestis and Pseudotuberculosis has also been reported to be depressed by arsenite (310). These findings do suggest, therefore, that the deamination of Dl-alanine by A.aceticgenum might be catalyzed by an amino acid oxidase system. Inhibition studies by carbonyl group reagents, which have lately been shown to be competitive inhibitors for L-amino acid oxidase of snake venom (311), also seem to favour the presence of an amino acid oxidase system.

8. An attempt was made to investigate the nature of the coenzyme involved in the deamination of Dl-alanine by A.aceticgenum. Only those vitamins and their analogues which were available, were tested. Pyridoxine, Pyridoxamine, pyridoxal phosphate and riboflavin phosphate failed to show any noticeable effect on the deamination of Dl-alanine when fresh or two months old cells of the organism, which had lost most of their deaminase activity, were used (Table No.XXVII).

Pyridoxamine, pyridoxine and pyridoxal phosphate even showed some inhibitory effect. Riboflavin which has been reported to show some stimulatory effect on the deamination of certain amino acids e.g. alanine, and phenylalanine etc. by mammalian kidney and liver; and mucosa homogenates of cow's intestine (289, 301) was not able to exercise a similar effect on the deamination of alanine by A.aceticgenum. Since cell free preparations were not used in these experiments, no satisfactory conclusion with respect to the coenzyme involved could be drawn from these observations.

9. Lichstein and Umbreit presented some evidence to show that biotin, in some form, was able to function as a coenzyme in the deamination of serine and threonine by Pr. vulgaris, Bacterium cadaveris and E.coli (208, 209). Other workers also obtained results which supported the involvement of biotin as a cofactor in the deamination of aspartic acid, serine and threonine by resting cells of bacteria (305, 317). Deamination of alanine, aspartic acid and glutamic acid by Neurospora mycelium was also shown to be activated by biotin (319). The results obtained in the present investigation (Table No. XXVI), however, indicated that biotin was not involved in the deamination of Dl-alanine or Dl-serine.

10. The metal binding agents also had an adverse effect on the deamination of Dl-alanine and Dl-serine. 8-hydroxy-quinoline and ethylene diamine tetra acetic acid (EDTA) appeared to be more potent inhibitors than α-α' bipyridyl in the case of both the amino acids. Complete inactivation of alanine and serine deaminases was brought about by 8-hydroxy quinoline and EDTA when they were kept in contact with cell suspensions for 4 hours and 3 hours respectively. However, with α-α' bipyridyl, after 4 hours, this was only about 73% in the case of alanine and 100% in the case of serine. As would appear from the results tabulated in Tables XXIV and XXV, the degree of inhibition in the case of both the amino acids was dependant on the concentration of these inhibitors and also on the time these inhibitors were in contact with the cell suspension. Serine deaminase appeared to be more susceptible to their action than alanine deaminase. These observations suggest that some metals might be involved in the deamination of alanine and serine by A.aceti gennm. Inhibition by cyanide also supports this assumption. Other amino acid deaminases in bacteria have shown to be associated with metal ions (136, 204) and were, therefore, inactivated in presence of metal binders. Amino acid deaminases from sources other than bacteria have also been reported to be metal dependant (300).

11. Inhibition of serine dehydrase (deaminase) by carbonyl group reagents has been reported by several workers in most of the microorganisms (131, 212, 220). The effect of hydroxylamine, sodium bisulphite, semicarbazide hydrochloride and sodium cyanide on the deamination of Dl-serine by *A.aceticanum* was, therefore, also studied.

From the results obtained, presented in Table No. XXIII, it would appear that these inhibitors did inhibit, to some extent, the deaminase activity but none of the inhibitors tested could completely inactivate the enzyme even at concentrations as high as 50 µ moles/ 3 ml. of the reaction mixture. Even sodium cyanide, the most potent out of these four, could bring about the inhibition of the deaminase activity to about 55.55% when added in a concentration of 50 µ moles/ 3 ml. of the reaction mixture.

12. Deamination of serine has been reported to be stimulated by pyridoxal phosphate (207, 220). Activation by glutathione and AMP(adenosine monophosphate) was observed with serine deaminase of *E.coli* (205). Anaerobic and reduced conditions have also been shown to favour the deamination of serine (255). Pyridoxine, pyridoxamine, pyridoxal phosphate, cysteine, and anaerobic conditions attained by gassing with

nitrogen were not able to exercise any appreciable effect on the deamination of both, Dl or L-serine, either with fresh cells, or when two months old cells which had lost most of their deaminase activity were used (Table No.XXVIII). Pyridoxal phosphate (PLP), however, was able to activate to a slight extent the deamination of Dl-serine when two months old cells of the organism were used. On the basis of these observations, it can be postulated that in the deamination of Dl-serine perhaps two enzymes, D serine deaminase and L-serine deaminase are involved. The former seems to be PLP dependant, and is inactivated in the presence of carbonyl group reagents, while the latter is not. This would also explain about 50% inhibition of serine deaminase activity in presence of carbonyl group reagents. Further, pyridoxine or pyridoxamine were not able to replace pyridoxal phosphate.

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